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From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

Comparison of the Lipolytic Activity of Circulating and Locally Released Noradrenaline during Acidosis

By

PAUL HJEMDAHL and BERTIL B FREDHOLM

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Abstract

HJEMDAHL P and B B FREDHOLM *Comparison of the lipolytic activity of circulating and locally released noradrenaline during acidosis* Acta physiol scand 1974 92 1—11

The influence of hypercapnic acidosis on the effects of i v noradrenaline (NA) infusions and of sympathetic nervous stimulation were studied in the isolated autoperfused subcutaneous adipose tissue of the dog Acidosis increased resting vascular resistance in the adipose tissue Infusion of NA (0.35—0.69 μg/kg/min for 30 min) increased adipose tissue blood flow at both pH 7.4 and 7.0 Arterial increases in glycerol and FFA due to NA infusions were inhibited by 60—70 % during acidosis Mobilization of these lipolytic products from the adipose tissue was impaired to the same degree Sympathetic nervous stimulation (4 Hz for 5 min) lead to the same increase in vascular resistance at normal and acid pH The lipolytic response i e glycerol release to nerve stimulation was unaffected by acidosis FFA release was however reduced by about 40 % Release of ^{3}H NA by nerve stimulation from previously labelled adipose tissue was not significantly increased during acidosis We conclude that inhibition of NA induced increases in arterial glycerol and FFA concentrations by acidosis can be explained by impaired mobilization from the adipose tissue Furthermore the lipolytic effect of locally released NA is not inhibited by acidosis in contrast to the findings with circulating NA This difference cannot to any major extent be explained by increased transmittor release during acidosis

In the isolated perfused subcutaneous adipose tissue of the dog vascular and metabolic effects of catecholamines or sympathetic nerve stimulation can be studied simultaneously (Ngai *et al* 1966 Fredholm 1970) The reactions of this tissue to hemorrhagic shock were studied by Kovach *et al* (1970) They found that in spite of the high sympatho-adrenal activity induced by hemorrhagic hypotension neither FFA release from the adipose tissue nor arterial FFA levels increase during bleeding Lactic acidosis is a constant finding in hemorrhagic shock Using the same adipose tissue preparation as Kovach *et al* and as is employed in this study Fredholm (1971) showed that local infusion of lactate inhibited FFA release by increasing the rate of reesterification provided arterial concentrations were above 5 mM The in

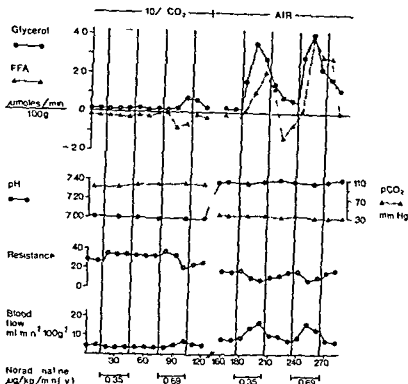


Fig 1 Effect of NA infusions in expt. 4 Dog weighed 15 kg and adipose tissue 0 g 55 min of equilibration with CO_2 before first experimental period 35 min of ventilation with air before control period Hematocrit 42—50 % Net release of glycerol and FFA are given in Table II

fluence of acidosis *per se* on this adipose tissue preparation has however not previously been investigated

In a study of the influence of acidosis on vasoconstriction in the cat hind leg Bygdeman (1963) noted a difference between locally released and blood borne NA We have therefore investigated the effect of acidosis on lipolysis stimulated by both NA infusions and sympathetic nerve stimulation in the subcutaneous adipose tissue

Methods and materials

The experiments were performed on 18 female mongrel dogs weighing 7—19 kg The dogs were anesthetized with sodium pentobarbital (30 mg/kg i v) with small supplements as required Tracheotomy was performed and the dogs were mechanically ventilated with a Braun Melsungen model 74052 respirator Muscle relaxation was maintained throughout all experimental runs by means of a slow i v infusion of succinylcholine chloride (Celokurin klorid Vitrum) amounting to approximately 10—15 mg/kg/h

Subcutaneous adipose tissue in the right inguinal region was isolated from skin and other surrounding tissues according to the method described by Rosell (1966) This provides an adipose tissue preparation supplied by one artery one vein and one mixed nerve suitable for electrical stimulation The weight of the preparation varied between 21 and 1?? g (average 37 5 g) Arterial blood was diverted from the femoral artery via a drop counter (Lindgren 1958) to the adipose tissue Venous outflow was returned to the femoral vein via a poly

TABLE I Increase in arterial concentrations (mM) at 25 min after start of NA infusions Mean ±1 S D from 5 expts with the low dose and from 6 expts with the high dose

	0.35 µg/kg/min		0.69 µg/kg/min	
	control	acidosis	control	acidosis
Glycerol	0.34±0.16 $p < 0.01$	0.08±0.08	0.39±0.15 $p > 0.005$	0.18±0.14
FFA	1.47±0.36 $p < 0.001$	0.33±0.2? $p < 0.05$	1.5?±0.48 $p < 0.001$	0.50±0.31 $p < 0.0?$
Glucose	0.6±0.8	−0.9±1.0	2.0±1.7 $p < 0.05$	1.5±1.0 $p < 0.0?$
Lactate	−0.12—0.25	0.08±0.11	−0.21±0.44	−0.?0±0.26
Pyruvate	−0.01—0.03	0±0.07	0±0.02	−0.0?±0.02

ethylene catheter containing a 3 way stopcock used for venous blood sampling Heparin 2500 IU/kg (which was kindly supplied by AB Vitrum) was administered before the cannulation procedure *i e* at least 45 min prior to the first experimental run Blood loss due to sampling was compensated by replacement with isotonic saline Systemic blood pressure was measured with Statham P23Ac transducers and recorded together with blood flow on a Grass model 7B polygraph Vascular resistance (in PRU) was obtained by dividing blood pressure with adipose tissue blood flow

The effects of hypercapnic acidosis have repeatedly been shown to depend on the increased H concentration rather than on hypercapnia *per se* (Bygdeman 1963 Nahas 1970) Therefore ventilation with 10 % CO_2 in 30 % O_2 balance N was chosen This is technically simple and gives reproducible pH changes Equipment from Radiometer (Copenhagen) was used for measurements of pH pCO and pO_2 In experiments 7—11 a continuous flow cuvette (DS 66014) fitted with appropriate glass calomel pCO and pO_2 electrodes was inserted into a loop from the left femoral artery to the corresponding vein In the remaining experiments intermittent determinations on arterial blood samples were made on a BMS 3 Mk 2 Blood Micro System equipped with similar electrodes Intermittent sampling gave somewhat more dependable values due to the possibilities for frequent calibrations Steady state was obtained after 30—60 min of CO ventilation and normalization of the pH after a period of acidosis required approximately the same time During control periods the pH was 7.35±0.03 pCO 33±3.5 mm Hg and pO 90±9 mm Hg During ventilation with 10 % CO the pH fell to 6.99±0.03 pCO_2 rose to 85±6 mm Hg and pO to 119±12 mm Hg

The nerve supplying the adipose tissue was sectioned in all experiments When stimulations were performed the nerve was placed on a bipolar silver electrode and protected from drying with Plastibase (Squibb) Stimulation impulses were delivered by a Grass model S4 stimulator equipped with an isolation unit (SIU-4B) 5 min stimulations at supramaximal intensity (12 V) and duration (2 ms) with a frequency of 4 Hz were chosen Venous blood from the adipose tissue was collected into ice cooled centrifuge tubes Aliquots of whole blood were removed for the determination of lactate and pyruvate (TCC TCB Boehringer & Sohn Mannheim) After centrifugation plasma was taken for the determination of glycerol (Laurell and Tibbling 1966) FFA (Trout *et al* 1960 or Laurell and Tibbling 1967) and glucose (with commercially available glucose oxidase reagent Glox, Kebo Stockholm) The two methods for determination of FFA yield indistinguishable results (Laurell and Tibbling 1967) The venous sampling followed a standardized pattern illustrated in Fig 3 Arterial samples were drawn immediately *before* and *after each* observation period for determination of hematocrit and the above mentioned metabolites

In 6 expts noradrenaline (L-arterenol HCl SIGMA) was infused into a separate forearm vein Two doses were chosen 0.35 and 0.75 µg/kg/min calculated as free base for 30 min The infusion speed was always 0.23 ml/min Noradrenaline was freshly prepared and the infusion syringe was kept cold by ice bags Blood samples were collected with the same technique as mentioned above except at different intervals (Fig 1) Arterial samples were frequent since arterial levels varied due to the systemic NA infusion Only glycerol and FFA were determined from venous samples in these experiments In addition lactate pyruvate and glucose were determined in arterial samples

In 5 expts. the adipose tissue was labelled with 1—7 ^{3}H NA (9.? Ci/mM Radiochemical Center Amersham) prior to nerve stimulation 100—?00 µCi ^{3}H NA was diluted to 10 ml in

TABLE II Total net release of glycerol and FFA caused by i v NA infusions expressed as μm l 100 g adipose tissue * indicates that acidosis preceded the control period

	Glycerol release				FFA release			
NA (μg/kg/min)	0 35		0 69		0 35		0 69	
Expt	control	acidosis	control	acidosis	control	acidosis	control	acidosis
1	111	41	124	76	5	− 3	47	30
2	60	7	69	12	47	4	108	5
3*	62	4	51	42	57	−10	52	54
4*	100	1	113	12	51	− 3	114	− 9
5*	—	—	41	13	—	—	22	3
6	143	15	274	51	103	− 2	211	10
Mean	95	14	112	34	52	− 3	92	17
S D	±31	±15	±79	±24	±31	±4	±63	±21
p		<0 005		<0 05		<0 02		<0 05

TABLE III Net glycerol and FFA release during 5 min of nerve stimulation and 10 min thereafter expressed as μmol/100g adipose tissue * indicates acidosis before control period, where two stimulations were performed

	Glycerol		FFA	
Expt	control	acidosis	control	acidosis
7	16 5	24 6	1 9	−2 2
8	22 5	24 8	39 5	24 7
9	3 8	3 6	3 3	2.8
10	9 8	11.2	3 5	6 4
11	4 6	1 2	—	—
12	7.9	7 4	33 3	10 4
13*	8 5	3 4	8 6	4 5
14	16 7	13 9	−4 3	−9 0
	11 1		11 0	
15		41 1		48 1
	44 5	35 3	43 8	18 6
16*	24 3	28 3	30 6	20 3
17	24 5	15 1	26 1	6 4
	18 8		12 0	
18	37 6	21 4	40 8	6 9
	23 2		14 9	
mean	18 3	17 8	18 9	11 5
S D	±11 3	±12 2	±15 7	±14 3
		NS		p < 0 00

isotonic salne ontaining 20 μg/ml ascorbic acid The solution was kept cold by ice bags while slowly 40—70 min being infused via a sidearm to the arterial cannula A washout period of 70—90 min was allowed before the first experimental run in order to obtain a steady basal outflow of radioactivity Blood samples were collected and analyzed as described for the other nerve stimulation experiments In addition 0 2 ml arterial and venous plasma samples were diluted in Instagel (Packard) and radioactivity was measured by a Packard liquid scintillation counter

Uptake or release rates for metabolites or radioactivity were calculated by multiplying the A—V difference by plasma flow in ml/min/100 g Basal conditions were usually detrmined

by two venous samples prior to a nerve stimulation or a NA infusion. Glycerol, FFA and ^{3}H net release were calculated by summing the total release during and following nerve stimulation or NA infusion and subtracting the basal release for the same time period. They are expressed as μmol/100 g and cpm/100 g respectively.

Results are expressed as mean values ± 1 S.D., unless otherwise stated. Statistical evaluation was performed with Student's t test for paired observations.

Results

Throughout this study the order of control and acidosis periods has been varied in order to eliminate systematic variations due to time and treatment. In 5 of the nerve stimulation experiments the control period preceded the acidosis period while the reversed procedure was employed in 3 expts. In 4 expts. however there were 3 periods. In half of the six NA infusion experiments acidosis preceded the control period (see Tables II and III).

Resting conditions. Blood pressure did not change significantly due to acidosis. However, in spite of denervation there was a significant ($P < 0.05$) increase in adipose tissue vascular resistance so that blood flow fell from an average of 7.8 ml/min/100 g (range 3.3—14.1) to 5.5 ml/min/100 g (range 1.3—16.2) during acidosis.

The arterial concentration of glycerol was 0.14 ± 0.07 mM during control conditions and was not significantly changed by acidosis. Arterial FFA levels decreased from 1.02 ± 0.45 to 0.67 ± 0.29 mM ($p < 0.001$) and glucose concentrations increased from 5.7 ± 2.6 to 7.6 ± 3.9 mM ($p < 0.05$) during acidosis.

There was a small but consistent increase in resting glycerol release from the adipose tissue from 0.22 ± 0.16 to 0.27 ± 0.13 μmol/min/100 g ($p < 0.05$) during acidosis. At a normal pH there was an FFA uptake amounting to 1.05 ± 1.15 μmol/min/100 g in the unstimulated adipose tissue. At pH 7.0, when the arterial levels were lowered, the FFA uptake decreased to 0.25 ± 0.37 μmol/min/100 g ($p < 0.01$). Glucose uptake and lactate and pyruvate release by the adipose tissue were not significantly altered by acidosis. The venous lactate/pyruvate concentration ratio was similarly unchanged by acidosis (23 ± 10 at normal pH and 30 ± 10 at pH 7.0, N.S.).

Noradrenaline infusions

Systemic responses. Blood pressure reached a peak within 5 min from the start of NA infusions. The low dose (0.35 μg/kg/min) gave a blood pressure maximum of 25 (7—60) mm Hg above resting levels and the high dose (0.69 μg/kg/min) raised it 50 (20—70) mm Hg. During acidosis the maximum pressor responses averaged 22 and 29 mm Hg respectively. They were not significantly different from the control responses. After the initial peak the blood pressure gradually returned towards but did not reach control values.

The effects on glycerol, FFA, glucose, lactate and pyruvate concentration in

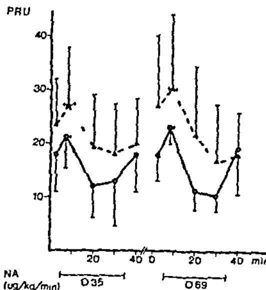


Fig 2 Vascular resistance during iv NA infusions Solid lines represent control values and dotted lines acidosis values Mean ± 1 S D from 6 expts

arterial blood caused by NA, are summarized in Table I Acidosis clearly inhibited systemic increases in glycerol and FFA (see also Fig 4) With the high dose of NA a hyperglycemic effect was seen which despite the raised basal level remained during acidosis Lactate and pyruvate did not change significantly

Adipose tissue responses The adipose tissue vascular resistance during intravenous NA infusions is shown in Fig 2 During control conditions, there is an initial increase in resistance with both doses of NA which coincides approximately in time with the blood pressure peak This initial vasoconstriction is gradually converted to a dilation Vascular resistance is lower than resting values from 10—15 min until the end of the infusion when the resistance rapidly returns to resting values When NA was infused at pH 7 0 the pattern of vascular resistance was essentially unchanged although the resting resistance was higher The return towards normal after the end of the infusion was more prolonged during acidosis ($p < 0.05$ at the high dose only)

Net release of glycerol and FFA from the adipose tissue due to NA infusions is shown in Table II Acidosis clearly inhibited mobilization of both of these lipolytic products from the adipose tissue (see also Fig 4) Even though there is a significant reduction in both adipose tissue blood flow and release of lipolytic products during acidosis there is a lack of correlation between these events ($r = 0.2940$ with all observations pooled)

Adipose tissue blood flow vascular resistance and lipolysis in a typical experiment is shown in Fig 1 This experiment illustrates the inhibitory effect of a low pH on lipolysis and since acidosis preceded the control period the reversible nature of this phenomenon

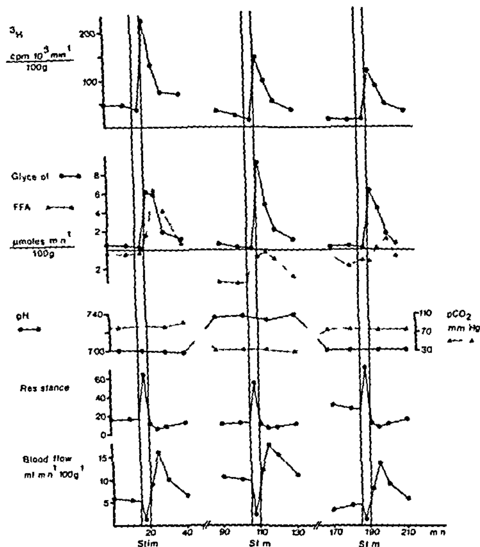


Fig 3 Effect of nerve stimulation in expt. 15 Dog weighed 11 kg and adipose tissue 26 g Hematocrit 35—40 %. Acidosis before and after control period 130 μCi of ^{3}H NA was infused during 40 min after which a wash out period of 80 min was allowed before the first experimental period Net release of glycerol and FFA are given in Table III and of radioactivity in the text

Nerve stimulations

The responsiveness of the adipose tissue to sympathetic nerve stimulation during acidosis was tested in 12 dogs A typical experiment is illustrated in Fig 3 During nerve stimulation the vascular resistance was increased from 19 5 ± 9 2 to 65 9 ± 40 6 PRU (327 ± 153 %) at the normal pH and from 28 2 ± 23 4 to 110 ± 90 3 PRU (389 ± 188 %) at pH 7 0 After stimulation there was a transient hype

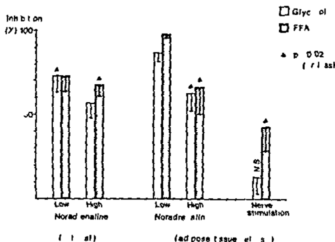


Fig 4 Comparison of the inhibitory action of acidosis on the release of glycerol and FFA due to circulating and locally released NA Arterial increases during i v infusions to the left. Mean values ± 1 S E from 5 expts with the low NA dose (0.35 μg/kg/min 6 expts with the high dose (0 69 μg/kg/min) and 19 expts with 5 min nerve stimulations

Minimal resistance at the normal pH was 13 8 ± 8 9 (70 4 ± 14 3 % of resting) compared to 14 3 ± 8 9 (57 0 ± 18 5 % of resting) during acidosis indicating that seen in relation to the resting levels of resistance the magnitude of the post stimulatory hyperemia is greater during acidosis ($p < 0.05$)

Net release of glycerol and FFA due to nerve stimulation is shown in Table III Glycerol release was not significantly altered by a pH of 7 0 whereas FFA release was inhibited by 43 % on the average (see also Fig 4) There was no correlation between adipose tissue blood flow and lipolysis ($r = -0.0657$) Release of ^{3}H noradrenaline was investigated in 5 of the 12 expts one of which is illustrated in Fig 3 In this experiment stimulation I (acidosis) yielded 0.86×10^6 cpm stim II (control) 0.59×10^6 cpm and stim III (acidosis) 0.67×10^6 cpm Thus there was a lower release of radioactivity during control conditions in this experiment Results were however variable and the pooled data show an insignificant increase amounting to 20 ± 31 % in release of radioactivity due to nerve stimulation at a pH of 7 0

Discussion

Intravenous infusion of NA increases arterial levels of FFA and glycerol (*cf* Hagen 1967) The response to 1 5 μg/kg/min of NA has been found to decrease by 50 % during acidosis (*cf* Nahas 1970) We found that a similar degree of acidosis inhibited the arterial response to infusions of 0 35 and 0 69 μg/kg/min by 70 and 60 % respectively Since a rise in arterial concentrations could be due to both an increased production and a decreased utilization of the metabolite in question the effect of acidosis may not entirely be due to a decreased mobilization from adipose tissue However as shown in Fig 4 the inhibition of the arterial response of FFA and glycerol was parallelled by an inhibition of mobilization of these metabolites

from our adipose tissue preparation of the same or even greater magnitude This finding also suggests that with regard to responses to acidosis the subcutaneous adipose tissue is representative for fat stores in general

During sympathetic nerve stimulation subcutaneous adipose tissue vascular resistance rises markedly (Ngai *et al* 1966) The situation is different following intravenous NA administration During NA infusion adipose tissue blood flow has been found to increase as measured by Xe disapperance in man (Nielsen *et al* 1968) microsphere technique in monkeys (Hoffbrand and Forsyth 1973) and direct measurement by dropcounter in dogs (Mjos and Akre 1971 Ballard 1973) We also found that after a brief period of increased vascular resistance adipose tissue blood flow increased during the NA infusion During acidosis resting vascular resistance increased in the adipose tissue in spite of denervation The responses to NA were however not significantly affected by acidosis It is therefore unlikely that the inhibition of lipolysis caused by acidosis is due to vascular factors which are known to modify metabolism in this tissue (*cf* Fredholm 1970) Furthermore there was no correlation between blood flow and lipolysis in either the nerve stimulation or the NA infusion experiments

In contrast to the situation during NA infusion we found no inhibition by acidosis of glycerol release induced by nerve stimulation (Fig 4) The vasoconstrictor response was also unaffected by hypercapnia When studying the effects of nerve stimulation the situation becomes more complex however since there are indications that transmittor overflow may be enhanced by acidosis Euler and Lishajko (1963) showed that acidosis accelerated depletion of NA from splenic nerve granules Nahas and coworkers (1967) found that catecholamine output from the isolated adrenal gland increased at a low pH Moreover acidosis has been shown to decrease NA uptake in mouse atria (Sachs 1970) The evidence for increased transmittor release *in vivo* during acidosis in dogs is only of an indirect nature and data are conflicting Morris and Millar (1962) found that in dogs ventilated with CO catecholamine levels increased with decreasing pH This finding was however not supported by Kohler and coworkers (1972) who found no significant increase in catecholamine levels during hypercapnic acidosis at a pH of 7 0 in this species

The different effects of acidosis on glycerol release induced by NA infusion (70 % inhibition) and by nerve stimulation (unchanged) could perhaps be explained by a compensatory increase in transmittor overflow during acidosis In order to test the possibility of increased transmittor overflow from adipose tissue nerve endings during acidosis the adipose tissue was prelabelled with ^{3}H NA and overflow of radioactivity in connection with nerve stimulations was studied according to a technique previously used in this tissue (Fredholm and Rosell 1970 Fredholm and Hedqvist 1973) We did not observe a significant increase in ^{3}H release during acidosis This indicates that if changes in transmittor release or uptake do occur during acidosis *the quantitative importance of this change is probably relatively small* An alternative explanation for the difference between blood borne and locally released NA

with regard to the inhibition of lipolytic activity by acidosis could be that the ac cessibility of the receptor to circulating and locally released NA may be affected in different ways by acidosis

Although glycerol mobilization following nerve stimulation was unaffected by acidosis a 40% inhibition of FFA release was observed It is interesting to note that resting arterial levels also fell during acidosis, in spite of an essentially unchanged glycerol concentration This could be due to increased re-esterification of fatty acids in adipose tissue during acidosis Lactate the concentration of which is elevated in shock causes increased re esterification (Fredholm 1971) There was no change in arterial levels of lactate in these experiments however The levels of glucose, which also can increase re esterification were raised during hypercapnic acidosis but it is not known if this is the cause of the observed changes in FFA

In conclusion we have confirmed earlier findings (*cf* Nahas 1970) that the elevation of plasma FFA and glycerol concentrations due to intravenously infused NA is inhibited by acidosis We have also shown an inhibition of the same magnitude of NA stimulated lipolysis in the subcutaneous adipose tissue which could not be explained by vascular factors In contrast to these findings acidosis did not significantly inhibit nerve stimulation induced glycerol release from the adipose tissue Hypercapnia did not significantly increase transmittor overflow due to nerve stimulation which leads us to believe that other factors are of importance in explaining this difference

This study was supported by grants from the Swedish Medical Research Council (40P 3878 40X 2553) and from Karolinska Institutet

We are grateful for the able technical assistance of Miss Lotta Malmstrom and Miss Margareta Stensdotter

References

BALLARD K Blood flow in canine adipose tissue during intravenous infusion of norepinephrine *Amer J Physiol* 1973 *225* 1026—1031

BYGDEMAN S Vascular reactivity i cats during induced changes in the acid base balance of the blood *Acta physiol scand 1963 61* Suppl *222*

EULER U S VON and F LISHAJKO Influence of pH on uptake and release of noradrenaline in adrenergic nerve granules *J Neurochem* 1963 *10* 145—149

FREDHOLM B B Studies on the sympathetic regulation of circulation and metabolism in isolated subcutaneous adipose tissue *Acta physiol scand* 1970 *80* Suppl 354

FREDHOLM B B The effect of lactate in canine subcutaneous adipose tissue in situ *Acta physiol scand* 1971 *81* 110—123

FREDHOLM B B and P HEDQVIST Role of pre and postjunctional inhibition by prostaglandin E_2 of lipolysis induced by sympathetic nerve stimulation in dog subcutaneous adipose tissue in situ *Brit J Pharmacol* 1973 *47* 711—718

FREDHOLM B B and S ROSELL Fate of ^3H noradrenaline in canine subcutaneous adipose tissue *Acta physiol scand* 1970 *80* 404—411

HAGEN J HIMMS Sympathetic regulation of metabolism *Pharmacol Rev* 1967 *19* 367—461

HOFFBRAND B I and R P FORSYTH Regional blood flow changes during norepinephrine tyramine and methoxamine infusions in the unanesthetized Rhesus monkey *J Pharmacol exp Ther* 1973 *184* 656—661

KOHLER, E E NOACK H STROBACH and K WIRTH Effect of acidosis on heart and circulation in cats pigs dogs and rabbits *Res exp Med* 1972 *158* 308—320

KOVACH A G B S ROSELL P SANDOR E KOLTAY E KOVACH and N TOMKA Blood flow oxygen consumption and free fatty acid release in subcutaneous adipose tissue during hemorrhagic shock in control and phenoxybenzamine treated dogs *Circulat Res* 1970 *26* 733—741

LAURELL S and G TIBBLING An enzymatic fluorimetric micromethod for the determination of glycerol *Clin chim Acta* 1966 *13* 317—322

LAURELL S and G TIBBLING Colorimetric microdetermination of free fatty acids in plasma *Clin chim Acta* 1967 *16* 57—62

LINDGREN P An improved method for drop recording of arterial and venous blood flow *Acta physiol scand* 1958 *42* 5—11

MJOS O D and S AKRE Effect of catecholamines on blood flow oxygen consumption and release—uptake of free fatty acids in adipose tissue *Scand J clin Lab Invest* 1971 *27* 221—225

MORRIS M E and R. A MILLAR, Plood pH/plasma catecholamine relationships respiratory acidosis *Brit J Anaesth* 1962 *34* 672—681

NAHAS G G The problems of acidosis *J clin Path* 1970 *23* Suppl 4 73—83

NAHAS G G and C POYART Effect of arterial pH alterations on metabolic activity of nor epinephrine *Amer J Physiol* 1967 *212* 765—772

NAHAS G G J C. LIGOU and B MEHLMAN Effects of pH changes on O_2 uptake and plasma catecholamine levels in the dog *Amer J Physiol* 1960 *198* 60—66

NAHAS G G D ZAGURY A MILHAUD W M MANGER and G D PAPPAS Acidemia and catecholamine output of the isolated canine adrenal gland *Amer J Physiol* 1967 *213* 1186—1192

NGAI S H S ROSELL and L. WALLENBERG Nervous regulation of blood flow in the subcutaneous adipose tissue in dogs *Acta physiol scand* 1966 *68* 397—403

NIELSEN S L V BITSCH O A LARSEN N A LASSEN and F QUAADE Blood flow through human adipose tissue during lipolysis *Scand J clin Invest* 1968 *22* 124—130

POYART C and G G NAHAS Inhibition of catecholamine induced calorigenesis and lipolysis by hypercapnic acidosis *Amer J Physiol* 1966 *211* 161—168

ROSELL S Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation. *Acta physiol scand* 1966 *67* 343—351

SACHS C Noradrenaline uptake mechanisms in the mouse atrium *Acta physiol scand* 1970 *79* Suppl 341

TROUT D L. E H ESTES JR. and S J FRIEDBERG Titration of free fatty acids of plasma a study of current methods and a new modification *J Lipid Res* 1960 *1* 199—202

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References

Ballard K Blood flow in canine adipose tissue during intravenous infusion of norepinephrine *Amer J Physiol* 1973 *225* 1026—1031

Bygdeman S Vascular reactivity in cats during induced changes in the acid base balance of the blood *Acta physiol scand* 1963 *61* Suppl 222

Euler, U S von and F Lishajko *Influence of pH on uptake and release of noradrenaline in adrenergic nerve granules* *J Neurochem* 1963 *10* 145—149

Fredholm B B Studies on the sympathetic regulation of circulation and metabolism in isolated subcutaneous adipose tissue *Acta physiol scand* 1970 *80* Suppl 354

Fredholm B B The effect of lactate in canine subcutaneous adipose tissue in situ *Acta physiol scand* 1971 *81* 110—123

Fredholm B B and P Hedqvist Role of pre and postjunctional inhibition by prostaglandin E₂ of lipolysis induced by sympathetic nerve stimulation in dog subcutaneous adipose tissue in situ. *Brit J Pharmacol* 1973 *47* 711—718

Fredholm B B and S Rosell Fate of ^{3}H noradrenaline in canine subcutaneous adipose tissue *Acta physiol scand* 1970 *80* 404—411

Hagen J Himms Sympathetic regulation of metabolism *Pharmacol Rev* 1967 *19* 367—461

Hoffbrand B I and R P Forsyth Regional blood flow changes during norepinephrine tyramine and methoxamine infusions in the unanesthetized Rhesus monkey *J Pharmacol exp Ther* 1973 *184* 656—661

Kohler, E E Noack H Strobach and K Wirth Effect of acidosis on heart and circulation in cats pigs dogs and rabbits *Res exp Med* 1972 *158* 308—320

twitch contractions of human muscle were calculated to test whether activation is partial or complete

Theory

In accordance with Hill (1949) the dynamics of muscular contraction were described in terms of a three element model consisting of i) a contractile component ii) an undamped series elasticity and iii) a parallel elasticity Considering isometric conditions only the presence of parallel elasticity can be disregarded The series elastic component was characterized by its force extension diagram which was assumed to be exponential (Wilkie 1950 Bahler 1967)

$$P = P_e \left(\exp(\lambda\ x_e) - 1\right) \tag{1}$$

where x_e is the extension in units of the unloaded muscle length L_0 P_e and λ are constants The slope of the force-extension curve of the series-elasticity i e its stiffness is according to (1)

$$\frac{dP}{dx} = \lambda\ (P+P_e) \tag{2}$$

In an isometric twitch the series elasticity is stretched at a rate equal to the shortening velocity V of the contractile component Therefore the rate of force development is

$$\frac{dP}{dt} = \frac{dP}{dx}\ V$$

or by inserting (2)

$$\frac{dP}{dt} = \lambda\ (P+P_e)\ V \tag{3}$$

Shortening velocity and force are related according to Hill's characteristic equation (Hill 1938)

$$V = \frac{P_0 - P}{P+a}\ b$$

In the present analysis the equation was modified to incorporate the active state curve

$$V = \frac{P_t - P}{P + a\ \frac{P_t}{P_0}}\ b\ \frac{P_t}{P} \tag{4}$$

where P_t is the intensity of active state i e the load which the contractile component is just able to carry without lengthening P_0 is the isometric tetanic force at the length considered and *a* and *b* are the constants of Hill's (1938) equation

Inserting (4) into (3) one obtains

$$\frac{dP}{dt} = \lambda\ (P+P_e)\ \frac{P_t - P}{P + a\ \frac{P_t}{P_0}}\ b\ \frac{P_t}{P}$$

Solving this equation with respect to $\frac{P_t}{P_0}$ leads to

$$\frac{P_t}{P} = \frac{1}{2}\left[\frac{P}{P} + \frac{\frac{a}{P}\ \frac{dP}{dt}}{b\lambda(P+P_e)}\right] + \sqrt{\frac{1}{4}\left[\frac{P}{P} + \frac{\frac{a}{P}\ \frac{dP}{dt}}{b\lambda(P+P_e)}\right]^2 + \frac{\frac{P}{P}\ \frac{dP}{dt}}{b\lambda(P+P_e)}} \tag{5}$$

From this expression the active state curve can be calculated if the following data are known the time course of isometric twitch tension (P(t)) its slope $\frac{dP}{dt}$ parameters *a* and *b* of the modified Hill equation (4) and parameters P_e and λ of the force-extension curve (1) of the series-elastic component

Experimental material

Recordings of normal and potentiated isometric twitches of normal subjects were put to my disposal by Dr A Slomic Dr A Rosenfalck and Dr F Buchthal (4 adductor pollicis muscles) and by Mr C Krarup (3 platysma muscles) * The experimental technique was

* I acknowledge with gratitude this material

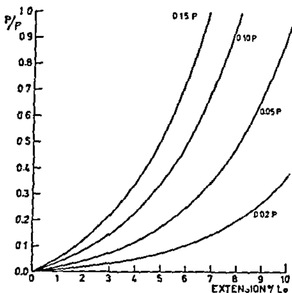


Fig 1 Force extension curves for the series elasticity of the two-element model of muscle used in calculating active state curves The curves are exponential $P = P_e$ (exp (l x_e) $-$ 1) where P is force and x_e is extension P_e and l are parameters characterizing the curves In the sample shown l 30 L_o^{-1} where L_o is the length of the unloaded muscle P_e is indicated on each curve in units of the isometric tetanic force P_0

described by Slomic *et al* (1968) with special reference to m adductor pollicis The muscles were stimulated supramaximally at a rate of 1 2 or 3 Hz for 90 s

Platysma was clamped at the mandible by a pair of forceps 5 cm broad and at the clavicula by another pair 1 or 3 cm broad and connected to a semiconductor strain gauge The muscle contractions were elicited by stimuli to ramus colli of n facialis Muscle action potentials were lead off from the endplate zone by a subcutaneous needle or an intramuscular wire

The twitches of the adductor pollicis muscles were recorded on photographic paper moving at a speed of 20 cm/s The records were enlarged 3 × to obtain sufficient resolution of the time course of development of tension The twitches of the platysma were photographed from a storage oscilloscope with a Polaroid camera the time base of the sweep being 20 ms per cm These records were magnified 3—4 ×

The isometric tetanic force of the platysma amounted to 540—6,0 g When twitches were recorded the oscilloscope was working at a sensitivity of 50 or 100 g/cm Hence 0 5 mm on the enlarged photograph corresponded to about 0 001 P_0 A similar resolution was obtained from the enlarged recordings from the adductor pollicis muscles

The force P(t) developed during normal and potentiated isometric twitches was read at intervals of 3 2 ms (m adductor pollicis with one exception of 6 2 ms) and 4 ms (platysma) on the enlarged recordings The rate of development of force $\frac{dP}{dt}$ was determined from these values

Inserting P(t) and $\frac{dP}{dt}$ in (5) the active state curve of the twitch was calculated It was assumed that l = 30 L_o^{-1} in the force-extension diagram (2) of series elasticity P_e was ordinarily assumed to be 0 05 P_0 other values ranging from 0 01 P_0 to 0 15 P_0 were tested as well Some of these force-extension curves are illustrated in Fig 1 With P_e = 0 05 P_0 the extension at P_0 is 0 10 L_o i e the same as found by *Wilkie* (1950) in the elbow flexors As to the parameters of the modified Hill equation (4) it was assumed (cf Wilkie 1950) that a = 0 33 P_0 and b = 1 L_o/s

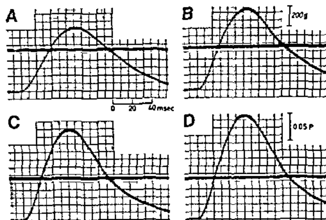
The calculations on isometric twitches of human muscle were supplemented by analysis of a normal and a post tetanically potentiated twitch response of rat muscle taken from Close and Hoh (1968 b) The curves were read at intervals of 1 2 ms It was assumed Close 1964) that a = 0 24 P_0 and b = 5 L_o/s

Results

a) m adductor pollicis

One normal (A) and three potentiated twitch responses (B C D) from trains of stimuli at 1, 2 or 3 Hz in one subject are presented in Fig 2 The active state

Fig 2 Normal (A) and potentiated (B C D) isometric twitches from m adductor pollicis of a normal subject U S 19 years old The contractions were elicited at rates of 1 2 and 3 Hz for 10 s by supramaximal stimuli to the *ulnar nerve at the* wrist. A first contraction B C D last contraction in train at 1 Hz 2 Hz and 3 Hz P tetanic tetanic force = 5 5



Velocities calculated from these twitches (Fig 3 A) show that in the normal twitch the intensity of the active state reached only about half of full activity In the potentiated twitches active state was intensified in the early phase of contraction the maximum activity amounting to 70—80 per cent of full activity The maximum *intensity was attained in 10—20 ms i e 20—35 per cent of the time to peak force* The active state curve of the potentiated twitch fused with that of the normal twitch as the peak force was approached

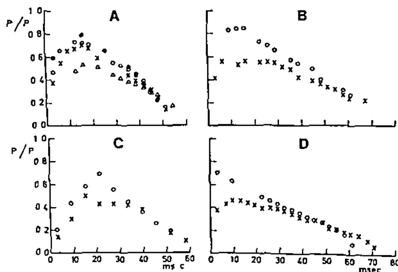


Fig 3 Sample of active state curves from normal and potentiated isometric twitches of m adductor pollicis in four normal subjects *A* refers to the twitches presented in Fig 2 △ 1 contraction × last contraction at 1 Hz ○ last contraction at 2 Hz ● last contraction at 3 Hz *B C D refer to 3 other normal subjects stimulated at 2 Hz* × *1 contraction* ○ *last contraction* In *B* the potentiation was 43 per cent in *C* 27 per cent and in *D* 33 per cent P intensity of active state It was calculated from (5) p 2 derived from Hill s two-element model of muscle with the series elasticity described by (1) p 2—and using the modified force velocity relation (4) p 2 The parameter P_e in (1) was assumed to be 0 05 P except in D where it was 0 07 P_o

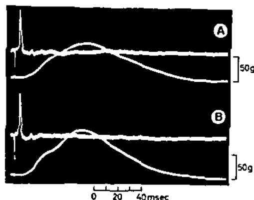


Fig 4 Normal (above) and potentiated (below) twitch contraction of platysma of a normal adult female A S stimulated at 1 Hz The potentiated twitch was no 115 Note the knee which becomes more prominent with potentiation P_o isometric tetanic force = 540 g The second beam of the oscilloscope presents the compound muscle action potential lead off in the endplate region

Similar findings were obtained in the 3 other subjects studied (Fig 3 B—D) In one subject it was assumed that $P_e = 0.02\ P_o$ i e a more compliant series element (Fig 1) since with $P_e = 0.05\ P_o$ the maximum intensity of the active state was only 27 and 37 per cent of the tetanic force for the initial and potentiated twitch

ii) *platysma*

In one subject the initial twitch response showed an early knee which became more prominent in the 30 per cent potentiated twitch (Fig 4) In the 2 other subjects in whom the potentiation amounted to 95 and 77 per cent there was a slight knee in the potentiated twitches These shapes probably reflect the activation of groups of fibres with different contraction times (Buchthal and Schmalbruch 1970) The active state curves calculated from the twitch responses gave support to this interpretation (Fig 5)

Potentiation was accompanied by intensification of the active state Whereas in the adductor pollicis muscle intensification was confined to the early phase of contraction in the two markedly potentiated twitches of platysma intensification lasted at least throughout the rising phase of the twitch (Fig 5 A B) In the third subject in whom potentiation was 30 per cent intensification of the active state exhibited two peaks at 10 and 35 ms (Fig 5 C)

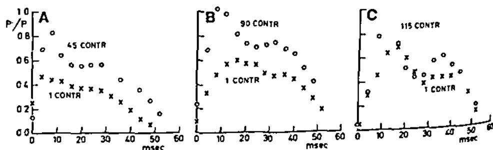


Fig 5 Sample of active state curves from normal (×) and potentiated (O) isometric twitches of platysma in three normal subjects stimulated at 1 Hz C refers to the twitches presented in Fig 4 where potentiation was 31 per cent In *A* and *B* potentiation was 95 and 77 per cent in contractions no 45 and 90 respectively P_i intensity of active state was calculated from (5) p 2 The parameter P_e of the series elasticity (*cf* ( ) p 2) was assumed to be 0 05 P in *A* and *C* and 0 10 P in *B*

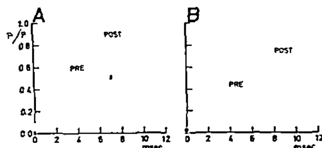


Fig 6 Active state curves of a normal (×) and a post tetanically potentiated (O) isometric twitch contraction of m. extensor digitorum longus of a rat, 4 weeks old (35 C) P_1 intensity of active state was calculated from (5) p ? using the experimental data of Close and Hoh (1968 b Fig 3 A and B) potentiation amounting to 80 per cent. The following assumptions were made for the parameters of the modified force velocity relation (4) p 2 $a = 0\ 24\ P_o$ and $b = 5\ L_o/s$ The parameter P_e of the series elasticity (1) p 2—was assumed to be 0 05 P_o in *A* and 0 10 P_o in *B* P_o isometric tetanic force was assumed to be 60 g corresponding to a twitch tetanus ratio of 0 21 as indicated by Close and Hoh (1968 b) in a table of average values

The influence of extensibility of the series element on the maximum degree of activation is illustrated in Table I for the 3 subjects An increase in P i e a *reduction in extensibility (Fig 1) caused a smaller degree of maximum activation* and a reduction in the intensification of activation during the staircase

iii) *m extensor digitorum longus of the rat*

The isometric twitch responses used in these calculations (Close and Hoh 1968 b Fig 3 A and B) were a control pre train contraction of m extensor digitorum longus of the rat and an 80 per cent potentiated response recorded 10 s after a train of 300 stimuli delivered in 1 s The contraction times of the 2 twitches were nearly the same 11 ms In the normal twitch the calculated active state curves had peaks of 55 and 70 per cent of full activation (Fig 6) depending on the stiffness of the series elasticity

TABLE I Influence of series elasticity on calculated peak of activation (P_1 max)

Subject	P /P	P_1^{max} /P		Potentiation (in per cent)	Twitch tetanus ratio
		normal twitch	potentiated twitch		
\	0 05	0 44	0 82	95	0 08
	0 10	0 30	0 53		
E F	0 05	0 81	(1 43)	77	0 20
	0 10	0 59	(1 02)*		
	0 15	0 50	0 84		
A S	0 05	0 70	0 97	31	0 13
	0 10	0 49	0 62		

* Values exceeding 1 are not acceptable physiologically

In the potentiated twitch active state was intensified throughout the phase of rising force the maximum activation being 88 and 99 per cent depending on the stiffness of the series elasticity (Fig 6)

Discussion

The present analysis of normal and potentiated isometric twitch contractions of mammalian skeletal muscle at or near body temperature showed that the staircase phenomenon and post tetanic potentiation are associated with an intensification of the active state. In normal (i e pre-train) twitches the early peak value of active state amounted to about 0.5 P_0 indicating that the contractile substance attained almost about half of full activation. The peak coincided with the maximum rate of rise of tension or was slightly earlier In the potentiated twitches 70—85 per cent of full activation was reached in m. adductor pollicis and 80—100 % in platysma.

The results support the explanation of the staircase phenomenon in human muscle put forward by Desmedt and Hainaut (1968) This explanation modified the current description of the activation process according to which full activity was attained abruptly in a twitch and was preserved for some time whereafter activity declined gradually (Hill 1949)

The main argument used by Desmedt and Hainaut (1968) was that the contraction time of the potentiated twitch was shortened. However two active state curves which start at full activity and coincide in the last part of the decline including the level of twitch tensions, can lead to twitch contractions in which the contraction time of the "potentiated" twitch is equal to or shorter than that of the "normal" twitch (Rosenfalck 1968)

To test the hypothesis of Desmedt and Hainaut it was necessary to determine the active state curve of normal and potentiated twitch contractions. In the present study these curves were calculated from the time course of tension development using Hill's (1949) two-component model of muscle. A prerequisite for the calculation was knowledge of (i) the load-extension diagram of the series-elastic component and (ii) the relationship between the velocity of shortening of the contractile component and the load throughout the twitch (cf p 2 It was assumed that this force-velocity relationship could be described by a modified Hill equation ((4 p 2 in which the active state curve was included

Bahler Fales and Zierler (1967) calculated an active state curve of mammalian muscle in a similar way They considered the contractile component to be a force generator shunted by a velocity sensitive internal load It was assumed that the internal load was the same in twitch and tetanus and that the force velocity relation was identical. In the examples presented by Bahler *et al* the maximum of the active state was within few per cent from full activity (rat's m. gracilis at 17.5° C twitch tetanus ratio 0.35—0 45)

This method of determining active state is likely to overestimate the degree of activation since the use of the force-velocity relation of tetanic contraction throughout the rising phase of the twitch must lead to internal loads which are too

because they correspond to a fully activated muscle Furthermore Bahler *et al* (1967) performed their experiments at low temperature when the maximum activation during the twitch is likely to be higher than near the temperature in the body (Close and Hoh 1968 a)

In the present analysis the force velocity relation was described by a modified Hill equation (4) p 2) whereas in the previous note (Rosenfalck 1968) a modified Aubert (1956) equation was used The simpler equation was chosen since the active state curves obtained with the 2 equations did not differ to any significant degree In the modified Hill equation the tetanic force P_0 was replaced by the intensity of the active state P_i and Hill s consant a by a/P_0 P_i This is equivalent to assuming that the heat of shortening per cm is proportional to the degree of activation Furthermore it was assumed that the velocity of free shortening was proportional to the intensity of the active state hence the velocity constant *b* was replaced by b P/P_0 Support for this assumption may be found in experiments on skeletal and cardiac muscle (Jewell and Wilkie 1960 Edman and Nilsson 1968 1969) as well as from contraction theories based on the sliding filament model (Julian 1969) It should be noted that active state curves calculated from a modified Hill equation when *a* was left unchanged differed only little from those presented in this paper The peak intensity of active state was reduced by maximally 0 06 P the time to peak being unaffected The initial decline in active state after the peak was somewhat faster than in Fig 3 the maximal difference amounting to 0 08 P_0

The present analysis was based on data from whole muscle There are indications that different fibres probably have different active state curves In single frog muscle fibres at low temperature (1—2° C) the duration of the active state varied by almost a factor of two from one fibre to another even at a fixed sarcomere length and the duration increased by 25—65 per cent with an increase in sarcomere length from 1 9 μm to 2 6 μm (Edman and Kiessling 1971)

An indirect indication of marked differences in the twitch response of different fibres is the large variation in the twitch tetanus ratio at resting length (0 15—0 74) and the finding that post tetanic potentiation is the more pronounced the smaller the ratio (Ramsey and Street 1941) Surprisingly a similar large variation in twitch tetanus ratio was seen at sarcomere length 2 1 μm in the frog s whole sartorius muscle at 20° C (Close 1972) Post tetanic potentiation with constant contraction time as observed in many fibres by Ramsey and Street (1941) has been taken as evidence of incomplete activation of the contractile component in the pre tetanic twitch (Close 1965) the degree of activation being directly correlated with the twitch tetanus ratio (Close 1972)

Data are not available on the distribution of contraction times and the range of the twitch tetanus ratio for the individual fibres of mammalian muscle near body temperature Several studies of single motor units have made it possible to distinguish between slow and fast motor units (*e g* Burke 1967) and within the group of fast units between fatigue susceptible and fatigue resistant units (Burke *et al* 1971) In m gastrocnemius of the cat (36—38°C) different fast motor units had twitch tetanus ratios of 0 17—0 71 (Burke 1967)

Such differences among motor units are likely to be reflected in their active state curves Hence, active state curves calculated from data on whole muscle can only represent some sort of an average for the individual fibres and motor units Data from single or very few fibres are therefore needed to further clarify the role of incomplete activation in the explanation of twitch potentiation

References

AUBERT X. *Le couplage énergétique de la contraction musculaire* Éditions Arscia Bruxelles 1956 315 p

BAHLER A S Series elastic component of mammalian skeletal muscle *Amer J Physiol* 1967 *213* 1560—1564

BAHLER A S J T FALES and K L ZIERLER The active state of mammalian skeletal muscle *J gen Physiol* 1967 *50* 2239—2253

BUCHTHAL F and H SCHMALBRUCH Contraction times and fibre types in intact human muscle *Acta physiol scand* 1970 *79* 435—452

BURKE R F Motor unit types of cat triceps surae muscle *J Physiol* (Lond) 1967 *193* 141—160

BURKE R F D N LEVINE F F ZAJAC P TSAIRIS and W K ENGEL Mammalian motor units Physiological histochemical correlation in three types in cat gastrocnemius *Science* 1971 *174* 709—712

CLOSE R Dynamic properties of fast and slow skeletal muscles of the rat during development *J Physiol* (Lond) 1964 *173* 74—95

CLOSE R The relation between intrinsic speed of shortening and duration of the active state of muscle *J Physiol* (Lond) 1965 *180* 542—553

CLOSE R E The relations between sarcomere length and characteristics of isometric twitch contractions of frog sartorius muscle *J Physiol* (Lond) 1972 *220* 745—762

CLOSE R and J F Y HOH The influence of temperature on isometric contractions of rat skeletal muscles *Nature* (Lond) 1968 a *217* 1179—1180

CLOSE R and J F Y HOH The after effects of repetitive stimulation on the isometric twitch contraction of rat fast skeletal muscle *J Physiol* (Lond) 1968 b *197* 461—47?

DESMEDT J E and K HAINAUT Kinetics of myofilament activation in potentiated contraction staircase phenomenon in human skeletal muscle *Nature* (Lond) 1966 *217* 529—532

EDMAN K A P and A KIESSLING The time course of the active state in relation to sarcomere length and movement studied in single skeletal muscle fibres of the frog *Acta physiol scand* 1971 *81* 182—196

EDMAN K A P and E NILSSON The mechanical parameters of myocardial contraction studied at a constant length of the contractile element *Acta physiol scand* 1969 *72* 205—219

EDMAN K A P and E NILSSON The dynamics of the inotropic change produced by altered pacing of rabbit papillary muscle *Acta physiol scand* 1969 *76* 236—247

HILL A V The heat of shortening and the dynamic constants of muscle *Proc roy Soc B* 1938 *126* 136—195

HILL A V The abrupt transition from rest to activity in muscle *Proc roy Soc B* 1949 *136* 399—420

JEWELL B R and D R WILKIE The mechanical properties of relaxing muscle *J Physiol* (Lond) 1960 *152* 30—47

JULIAN F J Activation in a skeletal muscle contraction model with a modification for insect fibrillar muscle *Biophys J* 1969 9 547—570

RAMSEY R W and S F STREET Muscle function as studied in single muscle fibers *Bio Symp* 1941 3 9—34

RITCHIE J M and D R WILKIE The effect of previous stimulation on the active state of muscle *J Physiol* (Lond) 1955 *130* 488—496

ROSENFALCK P Staircase phenomenon of human muscle relation to the active state *Nature* (Lond) 1968 *218* 958—959

SLOMIC A A ROSENFALCK and F BUCHTHAL Electrical and mechanical responses of normal and myasthenic muscle *Brain Res* 1968 *10* 1—79

WILKIE D R The relation between force and velocity in human muscle *J Physiol* (Lond) 1950 *110* 249—280

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From the Department of Physiology Gymnastik och idrottshögskolan Stockholm Sweden

Substrate Activation and Product Inhibition of LDH Activity in Human Skeletal Muscle

By

JAN KARLSSON BODIL HULTEN AND BERTIL SJODIN

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Abstract

KARLSSON J B HULTEN and B SJODIN *Substrate activation and product inhibition of LDH activity in human skeletal muscle* Acta physiol scand 1974 *92* 21—26

Substrate activation and product inhibition have been studied on LDH activity in both directions in human skeletal muscle K_m for the forward and backward reactions for pyruvate and lactate were found to be $0\ 8 \times 10^{-4}$ and $0\ 9 \times 10^{-2}$ M respectively For increases in concentrations of NAD and NADH the LDH activity was found to increase curvilinearly and no K_m value could be obtained Inhibition of the LDH activity corresponding to 50 % of V_{max} was obtained for the forward reaction with a lactate concentration equal to $0\ 3 \times 10^{-2}$ M and for the backward reaction (pyruvate) the corresponding value was $1\ 0 \times 10^{-4}$ M With concentrations of lactate and pyruvate approximately corresponding to maximal values observed in skeletal muscle with exercise the inhibition of LDH activity in both directions was equal to or more than 70 % of V_m It is concluded that in an *in vitro* system concentrations of lactate and pyruvate similar to what are observed under physiological conditions in human skeletal muscles will induce a significant product inhibition on LDH activity obtained from human skeletal muscles in both reaction directions It seems reasonable to assume that the present *in vitro* findings might reflect functions operating in an *in situ* system Thus it is possible that the products of the LDH catalysed reactions (lactat or pyruvate) might have a regulatory effect on the anaerobic glycolysis as well as the oxidation of lactate in human skeletal muscle

Human skeletal muscle possesses great capacities for anaerobic glycolysis The limiting factor for production of lactate is suggested to be either in the onset of the glycolysis (activation of the enzyme phosphorylase) or the phosphorylation on hexosmonophosphate residues (by the enzyme phosphofructokinase PFK) (for ref see Karlsson 1971) or both Later steps in the glycolytic pathway as *e g* reduction of pyruvate to lactate by the enzyme lactate dehydrogenase (LDH) has been neglected as to whether this step might exert a regulatory function in the reduction of pyruvate to lactate The reason has been that the LDH activity determined as V_m (optimal rate of reaction) has been found to be 3—5 times the highest rate of lactate formation observed (Karlsson 1971) Although the activity of LDH is far in excess of the maximal rate of lactate formation it does not necessarily mean that

Such differences among motor units are likely to be reflected in their active state curves Hence active state curves calculated from data on whole muscle can only represent some sort of an average for the individual fibres and motor units Data from single or very few fibres are therefore needed to further clarify the role of incomplete activation in the explanation of twitch potentiation

References

Aubert X. *Le couplage énergétique de la contraction musculaire* Editions Arscia, Bruxelles 1956 315 p

Bahler A S Series elastic component of mammalian skeletal muscle *Amer J Physiol* 1967 *213* 1560—1564

Bahler A S J T Fales and K L Zierler The active state of mammalian skeletal muscle *J gen Physiol* 1967 *50* 2239—2253

Buchthal F and H Schmalbruch Contraction times and fibre types in intact human muscle *Acta physiol scand* 1970 *79* 435—452

Burke R E Motor unit types of cat triceps surae muscle *J Physiol* (Lond) 1967 *193* 141—160

Burke R E D N Levine F E Zajac P Tsairis and W K Engel Mammalian motor units Physiological histochemical correlation in three types in cat gastrocnemius *Science* 1971 *174* 709—712

Close R Dynamic properties of fast and slow skeletal muscles of the rat during development *J Physiol* (Lond) 1964 *173* 74—95

Close R The relation between intrinsic speed of shortening and duration of the active state of muscle *J Physiol* (Lond) 1965 *180* 542—559

Close R E The relations between sarcomere length and characteristics of isometric twitch contractions of frog sartorius muscle *J Physiol* (Lond) 1972 *220* 745—762

Close R and J F Y Hoh The influence of temperature on isometric contractions of rat skeletal muscles *Nature* (Lond) 1968 a *217* 1179—1180

Close R and J F Y Hoh The after effects of repetitive stimulation on the isometric twitch contraction of rat fast skeletal muscle *J Physiol* (Lond) 1968 b *197* 461—477

Desmedt J E and K Hainaut Kinetics of myofilament activation in potentiated contraction staircase phenomenon in human skeletal muscle *Nature* (Lond) 1968 *217* 529—532

Edman K A P and A Kiessling The time course of the active state in relation to sarcomere length and movement studied in single skeletal muscle fibres of the frog *Acta physiol scand* 1971 *81* 182—196

Edman K A P and E Nilsson The mechanical parameters of myocardial contraction studied at a constant length of the contractile element *Acta physiol scand* 1968 *72* 205—219

Edman K A P and E Nilsson The dynamics of the inotropic change produced by altered pacing of rabbit papillary muscle *Acta physiol scand* 1969 *76* 236—247

Hill A V The heat of shortening and the dynamic constants of muscle *Proc roy Soc B* 1938 *126* 136—195

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Jewell B R and D R Wilkie The mechanical properties of relaxing muscle *J Physiol* (Lond) 1960 *152* 30—47

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Ramsey R W and S F Street Muscle function as studied in single muscle fibers *Biol Symp* 1941 *3* 9—34

Ritchie J M and D R Wilkie The effect of previous stimulation on the active state of muscle *J Physiol* (Lond) 1955 *130* 488—496

Rosenfalck P Staircase phenomenon of human muscle relation to the active state *Nature* (Lond) 1968 *218* 958—959

Slomic A A Rosenfalck and F Buchthal Electrical and mechanical responses of normal and myasthenic muscle *Brain Res* 1968 *10* 1—78

Wilkie D R The relation between force and velocity in human muscle *J Physiol* (Lond) 1950 *110* 249—280

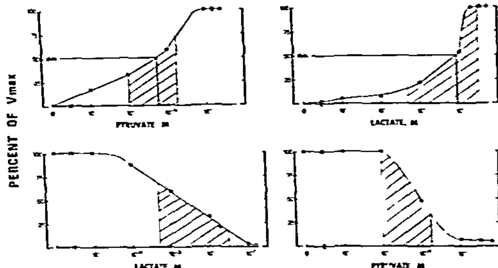


Fig 1 Effect of different concentrations of pyruvate and lactate as substrates or products on the LDH reaction in the forward (pyruvate reduced to lactate) or the backward reaction (lactate oxidized to pyruvate). The shaded areas indicate the normal concentration ranges for these metabolites in human skeletal muscle (Karlsson 1971). Each point represents the mean of not less than 3 separate analyses.

with test media made up with different pH, concentrations of substrates etc. will be expressed in percent of V_{max}. Incubation and fluorometer readings were performed either at room temperature (21 C) or at 35 C, and measurements of nonlinear relationships were made as previously described (Karlsson et al. 1969).

Results

The V_{max} data obtained for LDH activity ranged 0.6—1.4 × 10^{-4} and 0.3—1.1 × 10^{-4} mol × g^{-1} × min^{-1} in the forward and backward reactions respectively and were consequently in agreement with previous data (Karlsson et al. 1969). In respect to

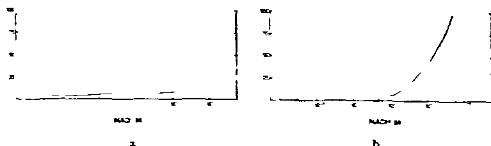


Fig 2 The effects of different concentrations of NADH and NAD on the activity of the forward and backward reactions (see Fig 1). Each curve is obtained from 6 or more means (see Fig 1).

TABLE I Highest observed muscle tissue lactate concentrations in healthy or diseased muscle of man

Conditions when the samples were obtained	Muscle lactate concentration mmol × kg^{-1} wet muscle	Reference
Untrained subjects short time maximal exercise	16.9	Karlsson 1971
Trained subjects short time maximal exercise	22.7	Karlsson 1971
Exhaustive isometric contractions	21.8	Karlsson and Ollander 1972
Cardiogenic shock	34.1	Karlsson *et al* 1972
Maximal exercise in patients with Anorexia nervosa	31.5	Thorén personal communication
Maximal exercise in patients with tetralogy of Fallot	30.5	Eriksson personal communication

an unlimited amount of lactate will be formed. On the contrary the highest values observed for lactate accumulation in skeletal muscle of man during muscular exercise (dynamic or isometric) or in different diseases (Table I) are in close agreement and might indicate a significant product inhibition at a level corresponding to 20–30 mmol × kg^{-1}.

The aim of the present study was therefore to reinvestigate and to extend the knowledge about the kinetics of the enzyme LDH and to further define the properties of this enzyme in human skeletal muscle.

Methods and material

Needle biopsy material (Bergstrom 1962) were obtained from subjects who from previous studies were known to possess about equal distribution of fast twitch and slow twitch muscle fibres (for further information see Gollnick *et al* 1972) in their examined muscle (vastus lateralis). The biopsy material was stored frozen (below −80 C) until further analyzed. After thawing and sonification in 200 µl of 0.1 M Tris pH 7.5 the homogenate was centrifuged and exempted on fibre debris. The supernatant was then further diluted with 0.1 Tris buffer pH 7.5 to a final volume giving a dilution of 1:5000 (w/v). This dilution was used over a period of 2–3 days for different studies of LDH kinetics. Every day V_{max} of the homogenate was reassayed in both directions to ascertain any activity loss of the tissue homogenate that could endanger the interpretation of the results.

LHD activity was assayed in both directions, i.e. reduction of pyruvate (forward reaction) and oxidation of lactate (backward reaction) with a methodological error of less than 1%. The basic medias were 0.02 M Imidazol buffer pH 7.5, 1×10^{-3} M pyruvate and 1×10^{-6} M NADH for the forward reaction and 0.1 M Tris buffer pH 8.9, 2×10^{-2} M lactate and 1×10^{-3} M NAD for the backward reaction. The reactions were run on Farrand photoelectric fluorometer as earlier described (Karlsson, Diamant and Saltin 1968). Different pH levels were obtained with addition of either HCl or NaOH. Varying concentrations of substrates as well as reaction products of the test medias were obtained by addition of the above mentioned chemicals. They were taken from the same batches from which the basic test medias were made up. The chemicals used were of highest purity and obtained either from Sigma Chemical Co. or Boehringer Mannheim GmbH. The pH of the basic test medium for either reaction direction as well as concentrations of substrates were chosen to give a high rate of reaction (V_{max}). The similarly obtained LDH activity was set to 100%. Results obtained

found at a physiological pH level Increased lactate concentration in the medium *i e* product inhibition) shifted the peak further to the basic side Moreover at a pH level corresponding to 7 0 and 20 mM of lactate the product inhibition present above was further augmented For the backward reaction the activity seemed to increase the higher the pH Different levels of substrates (lactate), or products (pyruvate) did not change this general pattern although the curves were on a lower level (Fig 3)

The above presented results were obtained at room temperature To ascertain that no changes were related to a difference in temperature between the *in vitro* experiments (21° C) and the expected temperature *in situ* in the muscle additional readings were made for product inhibition at 37° C Practically identical results were obtained for the inhibitory effect of the products in the forward as well as the backward direction indicating that the temperature difference had no other effects than those expected from the increase in temperature per se

Discussion

The major findings from the present study are that in an *in vitro* system both the reduction of pyruvate (the forward reaction) and the oxidation of lactate (the backward reaction) are operating at the optimal part of the affinity curve as interpreted from the K_m values obtained Moreover at low concentrations of lactate as well as of pyruvate a significant product inhibition were present Although these results are derived from *in vitro* experiments and crude muscle biopsy homogenates it seems reasonable to assume that the results might be extrapolated to the *in situ* system and the living cell

It is well documented that human skeletal muscle contains all the 5 LDH isozymes in a pattern which is proportional to the percent slow twitch fibres of the mu cle (Karlsson *et al* 1974) Thus in a muscle with a high percent slow twitch fibres the major portion of the total LDH activity is attributable to the more heart specific LDH isozymes whereas in a muscle with a low percent of slow twitch fibres it is vise versa Moreover with an increasing number of slow twitch fibres the total LDH activity in both directions is decreased

As these different LDH isozymes possess different affinities for substrates (or demonstrate different substrate inhibition) (for ref see Sund 1968) the present results have to be interpreted with the above mentioned knowledge as a background It might be argued that the present data are obscured by these facts Although this to a certain extent is true it cannot be denied that the present data will exhibit a general physiological pattern which might be modified for the individual muscle in respect to the actual muscle fibre composition

References

BERGSTRÖM J Muscle electrolytes in man *Scand J clin Lab Invest* 1962 Suppl 68
KARLSSON J B DIAMANT and B SALTIN Lactate dehydrogenase activity in muscle after prolonged severe exercise in man *J appl Physiol* 1968 25 88—91

KARLSSON J Lactate and phosphagen concentrations in working muscle of man *Acta physiol scand* 1971 Suppl 358
KARLSSON J and B OLLANDER Muscle metabolites with exhaustive static exercise of different duration *Acta physiol scand* 1972 *86* 309—314
KARLSSON J J T WILLERSON S J LESHIN C B MULLINS and J H MITCHELL Skeletal muscle metabolites in patients in cardiogenic shock or congestive heart failure *Circulation* 1972 *46* Abstr 420
KARLSSON J K FRITH B SJÖDIN P D GOLLNICK and B SALTIN Distribution of LDH isozymes in human skeletal muscle *Scand J clin Lab Invest* 1974 In press
SUND H The pyridine nucleotide coenzymes In *Biological Oxidations* 1968 Interscience Publ New York 603—639

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From the Department of Physiology University of

Firing Behaviour of a Neurone Model Based on Afterhyperpolarization Conductance Time Course Algebraical Summation Adaptation and Steady State

By

F BALDISSERA[1] and B GUSTAFSSON

Received 19 February 1974

Abstract

F BALDISSERA and B GUSTAFSSON *Firing behaviour of a ... the afterhyperpolarization conductance time course and Adaptation and steady state firing* Acta physiol scand

A preceding paper described the firing behaviour of a neurone ... toriness was solely governed by a K conductance process similar ... hyperpolarization in the cat's α motoneurones (Baldissera and ... paper describes the firing behaviour of the same model implem... mation of the consecutive AHP conductance For the initial ... stant current the model is well simulating the successive ... f/i) relations and the interspike voltage trajectories found in ... state f/i relation of the model can also display an upward ... current intensities i e a primary and a secondary range of f/i ... neurones (Kernell 1965 c) The results are discussed in relation ... firing behaviour in motoneurones and to various hypotheses on ... that the AHP conductance is the decisive factor for determining ... the interspike voltage trajectories in motoneurones

In preceding papers (Baldissera and Gustafsson 1974 a ... model in which the repetitive firing is governed by the ... lying the afterhyperpolarization (AHP) This model well ... trajectorie and the shape of the f/i (frequency current) ... val of repetitive firing induced by step current injection ... of the cat (Baldissera and Gustafsson 1974 b)

In real motoneurones after the onset of a current ... the firing frequency (adaptation) which is complete ... spike intervals (Granit *et al* 1963 Kernell 1965 a) A ... sidered to be of large importance for this adaptation ...

[1] Present address Laboratorio di Biologia Spaziale ... 20131 Milano Italia

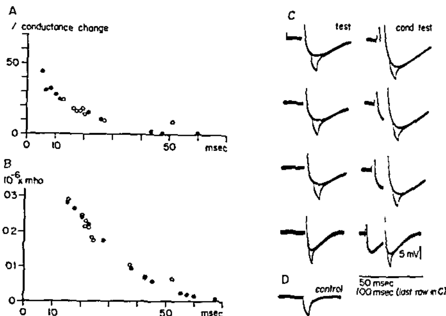


Fig 1 Conductance summation measured by current pulses or by computation from the AHP voltage Lumbar motoneurone spike size 85 mV Same neurone as in Fig 1 B (Baldissera nad Gustafsson 1974 a) A The conductance increase during the AHP following a single spike (measured by current pulses and expressed in % of the resting value) is plotted (filled circles) on the ordinate against the interval between the spike onset and the end of the current pulse The open circles represent the difference between the percentage conductance increase at the peak of the 2nd AHP when conditioned and when unconditioned by a preceding spike at the intervals plotted on the abscissa (see text) B Same procedure as in the plotting in A but performed for the same neurone with the AHP conductance computed from the AHP voltage C and D Records used for the plotting in A and B

and Oshima 1962 Baldissera and Gustaf son 1971 Baldissera *et al* 1973 Calvin and Schwindt 1972 Kernell and Sjoholm 1973) a term denoting the fact that when two successive spikes follow each other with an interval shorter than the AHP duration the peak voltage and conductance of the second AHP are larger than those of the first one We will in this paper give some evidence that the AHP summation in motoneurones as a first approximation can be simulated by an algebraical summation of successive AHPs The firing behaviour of the neurone model with an algebraical summation is subsequently computed and compared with that of real neurones It will be demonstrated that this implemented model displays an adaptation fairly similar to that of real motoneurones

A short preliminary report of some of the present results has been published (Baldissera and Gustafsson 1971)

Methods

For description of the methods see Baldissera and Gustafsson 1974 a b

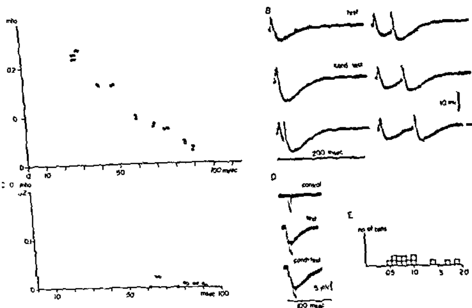


Fig 2 Computed conductance summation for two other motoneurones A Lumbar motoneurone spike size 100 mV Same procedure as in Fig 1 B Note that the summation is considerably larger than algebraical (see text) over the whole time course B Some of the records used for the plot in A C Motoneurone spike size 90 mV The summation in this neurone is considerably less than algebraical during most of the time course D The records are showing conductance and voltage summation for another neurone at a short interspike interval In this neurone both the voltage and the conductance summations were only around 50 % of the algebraical E Distribution of the correction factor (see text) in 13 neurones

Results

Section I AHP summation

The summation of the AHP conductance changes following successive spikes has been investigated both by direct measurements of the membrane conductance with the pulse technique and by calculating the membrane conductance from the AHP voltage under the assumptions previously stated (Baldissera and Gustafsson 1974 b)

We studied the time course of the summation of the AHP conductance by measuring the conductance at the peak of the AHP following the second of 2 antidromic spikes at different interspike intervals (Fig 1 C) In Fig 1 A the filled circles represent the time course of the conductance after a single spike The open circles give the difference between the conductance at the peak of the second AHP when conditioned by the first spike and the conductance at the same point in unconditioned situation This difference represents the increase in the conductance of the second AHP due to the conditioning effect of the first spike The abscissa gives the interval between the first spike and the end of the test pulse for both curves Observe that over a large part of the time course the increase in the conductance at the peak of the second AHP is equal to the conductance due to the first AHP at

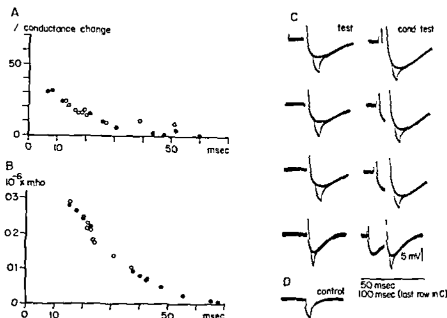


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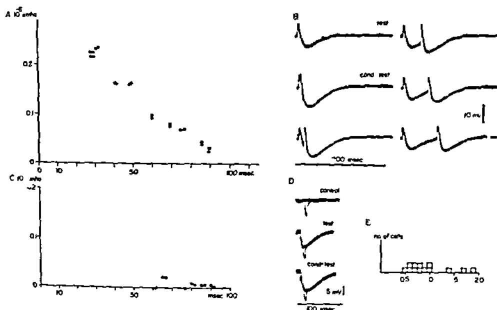


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that moment, i.e. the increase is compatible with a simple algebraical summation of the conductance of the first AHP to the conductance of the second AHP. Due to the unavoidable delay of the test pulse from the second spike, measurements are lacking in the early phase of the interaction curve.

In Fig. 1 B the conductance variations computed from the AHP voltages following one and two spikes are plotted in the same way as the experimental values of Fig. 1 A. The filled circles give the time course of the conductance variations after the first spike while the open circles represent the conditioning effect of the first spike on the conductance at the peak of the second AHP. Note that the computed conductance seems also to summate algebraically over a considerable time range.

In Fig. 2 A are plotted as in Fig. 1 B the values obtained in another neurone and in 2 B are inserted some of the records used for that plot. In this cell the computed conductance at the peak of the second AHP is much larger than the sum of the conductance of each single AHP. Note that the deviation from a simple summation is of the same order of magnitude over most of the time course. Fig. 2 C exemplifies a cell in which the summation was less than algebraical during most of the time course. See also the inserted records from another neurone in Fig. 2 D. In none of the neurones with less than algebraical summation at short interval was there any longlasting "depression" of the second AHP as found in dorsal spinocerebellar tract neurones (Gustafsson and Zangger 1974).

In three other cells the conditioned time courses were as in the neurones illustrated, running rather in parallel to that of the first AHP but with slight deviation from what expected by algebraical summation.

The summation can then be expressed by an algebraical summation multiplied by a correction factor which is assumed at a first approximation to be largely independent of the conditioning test interval. To evaluate the possible variability of the correction factor in different neurones the ratio

$$\frac{\text{conditioned conductance—unconditioned conductance}}{\text{unconditioned conductance}}$$

at the peak of the second AHP and at the shortest possible conditioning test interval was evaluated in 13 neurones by G_K t computation. This ratio would then be equal to and give a correction factor of 1 if the summation is algebraical. For the 13 neurones examined this factor covered a range from 0.51 to 1.85 with a mean value of 0.99 (see histogram).

We will study the adaptation in our model by applying the simplest expression for the AHP summation, i.e. by assuming algebraical summation.

Section II. Initial adaptation in the model

The criterion. The first interspike interval of repetitive firing of a motoneurone is satisfactorily computed by a model in which firing is governed by the AHP conductance changes (Baldissera and Gustafsson 1974 b). The same model can be utilized to predict the duration of the succeeding intervals with the further con-

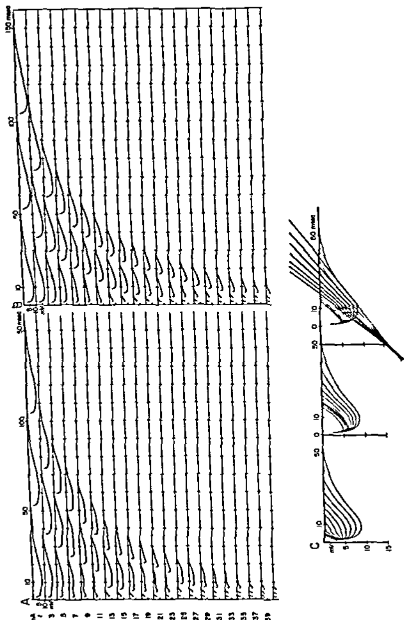


Fig 3 AHP *trajectories in the neurone model for the first three intervals during firing* A Model version with a local minimum local maximum conductance time course as in Fig 3 A (Baldissera and Gustafsson 1974 b) B Model version with a continuously decaying conductance time course as in Fig 3 B (Baldissera and Gustafsson 1974 b) C 1st 2nd and 3rd interval trajectories in B are shown superimposed for each interval on a common starting point. *The lines fitting the slopes of the ramps for the* 3rd interval trajectories are drawn by eye (see Discussion)

dition that the conductance changes after successive spike summate algebraically By solving in V equation 2 (see Baldissera and Gustafsson 1974 b) for a given $G_K(t)$, the trajectory of the first AHP is obtained When the AHP voltage crosses the threshold line ($V = 0$) a $G'_K(t)$ identical to $G'_K(t)$ is started and its value summed to the residual value of $G_K(t)$ This new conductance $G_K(t)+G_K(t)$ gives rise to the trajectory of the second AHP The second interval is then measured and the computation can be continued up to the desired number of intervals The initial conditions for V and t are the same as described previously i e the computation for each interval is started at a fixed voltage below the threshold and at a fixed time after the onset of the $G_K(t)$

In Fig 3 A and B are illustrated the voltage trajectories for the first 3 intervals of repetitive firing as computed by the model The time courses of $G_K(t)$ differ in A and B only in the plateau region and are the same used in a preceding paper (Baldissera and Gustafsson 1974 b) for describing the changes in the first interval of repetitive firing and are intended to represent the two general types of $G_K(t)$ time courses (Baldissera and Gustafsson 1974 a) In Fig 3 C are shown the trajectories for the first second and third interval given in Fig 3 B superimposed for each interval with the respective spike onset as a common starting point It can easily be observed that the second and third interval trajectories undergo the same general sequence of changes as that previously demonstrated and discussed for the first interval There are however some clear differences from the first interval 1) For a given interval length the scoop is more rounded or the upward convexity (or hump) is less expressed for the second and third interval Expressed according to the terminology of Schwindt and Calvin (1972) the primary range trajectory behaviour is reached at higher frequencies for the later intervals This behaviour is thus completely in line with the demonstration in an earlier report (Baldissera and Gustafsson 1974 b) that in real motoneurones the transition to secondary range trajectories took place for the first interval firing already well within the primary range of firing while the transition took place appreciably later for the following intervals 2) The change in ramp slope with increasing current which was not so evident for the first interval (see Fig 3 C) becomes more visible in later intervals being more pronounced for the lower current intensities

It can be observed in Fig 3 A and B that the trajectories for the 2nd and 3rd intervals display a complex series of changes with increasing current intensity The more complex behaviour occurs when the first interspike interval decreases below time to-peak AHP and is most pronounced in the neurones with the local minimum maximum plateau $G_K(t)$ time course (Fig 3 A) It can be noted that when the first interval jumps over the $G_K(t)$ plateau (11 to 13 nA) the second interval trajectory gets an appreciably more rounded scoop than for the preceding current value while the third interval instead gets a more upward convex or inverted scoop Note also the longer duration of the second interval than that of the third (13 to 19 nA) With increasing currents the second interval trajectory displays the same sequence of secondary range trajectory changes previously displayed by the

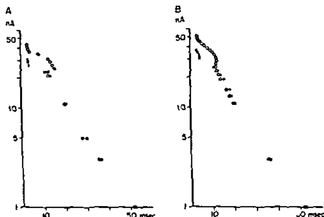


Fig 4 Relation between current injected and the duration of the interspike interval for the three first intervals for the computation shown in Fig 3 A and B In the graph are plotted the durations of the 1st interval (filled circles) and 2nd interval (crosses) and the 3rd interval (open circles) on the abscissa against the current injected on the ordinate (see text)

first one *i e* the scoop flattens and reverses to an upward convexity When the second interval decreases to a short value (25 nA) the third interval trajectory scoop expands temporarily and then reverses again with increasing currents as did the second interval before

I/i and f/i relations The i/i and f/i relations for the firing produced by the two different ($G_K(t)$ time courses computed in the preceding section are shown respectively in Fig 4 and 5 In Fig 4 are shown the i/i relations for the 1st (filled circles) the 2nd (crosses) and the 3rd (open circles) intervals Note that the close similarity between the firing curve for the first interval and the $G_K(t)$ time course after a single spike which was visualized in a preceding paper (Baldissera and Gustafsson 1974 b) is lost when considering the curves for the following intervals Because of the summation of the $G_K(t)$ the relations for the 2nd and the 3rd intervals assume a more complex time course which will be discussed into detail when represented on the f/i plot

On the other hand as for the trajectories the general behaviour of the 2nd and 3rd interval f/i relations in Fig 5 are very similar for both model versions to that of the first interval *i e* the curves display a primary secondary and tertiary range of firing Note however that the transition frequency between the primary and secondary range increases with the succeeding intervals approaching the 1/time to peak AHP (arrow on the ordinate) for the 3rd interval Observe in A that in the primary range the f/i relations for the 2nd and the 3rd intervals display more complex curves than that for the first interval When the first interval enters the secondary range the curve for the 2nd interval drops down and crosses the curve for the 3rd interval With further current increments while the first interval is in the tertiary range the curve for the second interval bends upwards and after a second crossing of the 3rd interval curve enters the secondary range At this moment the third interval curve drops down and the same sequence is *repeated* A similar but less marked behaviour is shown also by the other model version in Fig 5 B

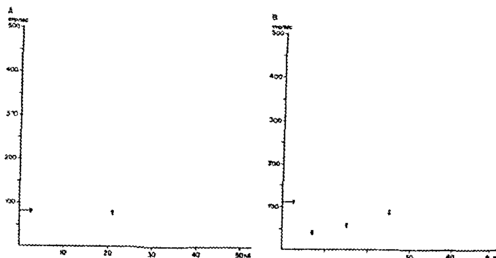


Fig 5 Relation between the current injected and the reciprocal of the three first intervals for the computations shown in Fig 3 A and B In the graph are plotted the reciprocal of the 1st interval (filled circles) 2nd interval (crosses) and 3rd interval (open circles) on the ordinate against the current injected on the abscissa (see text)

It should be noted that the sequence of changes in the f/i curves is directly related to the complex pattern of trajectory changes earlier described The dropping down of the f/i relations for the 2nd and the 3rd intervals is in fact related to the expansion of the scoop (Fig 3 A 13 nA for the 2nd interval and 25 nA for the 3rd interval) and the subsequent rise towards the secondary range to the inversion of the scoop (Fig 3 A from 17 to 23 nA for the 2nd interval and from 27 to 35 nA for the 3rd interval)

Steady state f/i relation The f/i relation for the steady state firing can then be derived by a continuation of the above computations until the adaptation is completely over To simplify the calculation the membrane capacitance was not taken into account This procedure seemed justified since the capacitance only gives a temporal shift of the interval current relation without affecting its general shape (Fig 3 Baldissera and Gustafsson 1974 b)

In Fig 6 are plotted the steady state f/i relations computed with two different $G_K(t)$ time courses one displaying a simple exponential decay (crosses) and the other an exponential decay interrupted by a local minimum maximum plateau (filled circles) For the first interspike interval *i e* before adaptation (or algebraical summation) both these time courses give rise to upward concave f/i relations displaying primary and secondary ranges of firing (*Baldissera and Gustafsson 1974 b* Kernell 1968 Kernell and Sjoholm 1973 MacGregor and Sharpless 1973) However after adaptation the resulting f/i relations differ from one another from this point of view The f/i relation given by a simple exponential decay of the $G_K(t)$ is now well fitted by an approximately straight line *i e* displays only a primary range This approximation to linearity is not only true in the frequency range

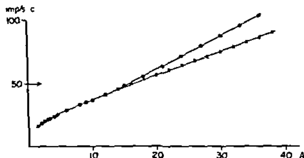


Fig 6 Steady state f/i relations in the neurone model The relation between the steady frequency (measured at the 20th interval for each current strength) on the ordinate against the current injected on the abscissa for two different $G_K(t)$ time courses (see text) The equations were $G_K(t) = 0.95 \times EXP\ (-t/20)$ (crosses) and $G_K(t) = 1.8 \times EXP\ (-t\ 2\ 1/2\ 0) + 0.95 \times EXP\ (-t/20) - 0.55 \times t^{0.08} \times EXP\ -t/20 \times EXP\ (-t\ 2\ 5/800)$ $V_K = 30$ Arrow marks the frequency at 1/time to-peak AHP Lines fitted to the curves by eye

illustrated but will extend to indefinitely high frequencies (see also MacGregor and Sharpless 1973) The steady state f/i relation given in the presence of a $G_K(t)$ plateau (filled circles) is on the other hand not approximated by a straight line but is deviating upwards at higher current intensities i e displays a primary and a secondary range as the initial intervals before Observe that the transition frequency between the two ranges is close to the frequency corresponding to 1/time to-peak AHP (arrow) It is thus clear that the presence of a pronounced $G_K(t)$ plateau is securing also for the steady state firing a transition to a secondary range before exceeding the 1/time to peak AHP In the lower frequency range it can be noted that both curves deviate from linearity with a downward bending when approaching zero current

Section III Comparison with real motoneurones

The firing produced by the model is thus very similar to that earlier described for motoneurones not only for the first interval (see Baldissera and Gustafsson 1974 b) but also for the following intervals i e one can identify the 3 firing ranges described by Kernell (1965 b) and by Schwindt (1973) and the trajectory changes described by Schwindt and Calvin (1972) As for the first interval there are also for the later intervals some aspects of the model firing not earlier recognized in motoneurones such as the sequential changes in the i/i and f/i relations and in the trajectories associated with the adaptation

F/i relations The sequential changes in the f/i relations for the 2nd and the 3rd interval firing were an invariable finding in more than 50 neurones examined in this respect and are illustrated for two neurones in Fig 7 A and B Note the clear similarity between the f/i relations from these neurones with those shown in Fig 5 for the computed firing In the f/i plot in Fig 7 B it can be observed that the jump present for the 1st interval (same neurone as in Fig 7 Baldissera and Gustafsson 1974 b) is not found in the 3rd interval f/i relation This behaviour is then in contrast to the model behaviour where the jump is also present for the 3rd interval

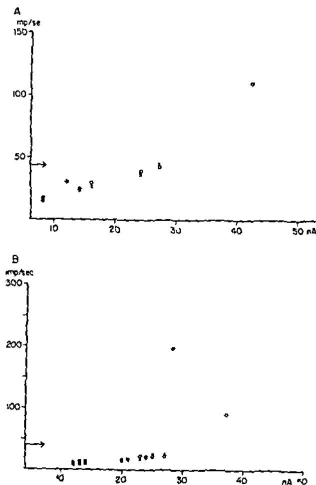


Fig 7 Current frequency curves for the first three intervals in lumbar motoneurones A Lumbar motoneurone spike size 100 mV In the graph are plotted the reciprocal of the 1st interval (filled circles) 2nd interval (crosses) and 3rd interval (open circles) on the ordinate against the current injected on the abscissa. The plotted values are the mean of 2 trials B As in A but for another neurone Spike size 85 mV The plotted values are the mean of 3—4 trials The arrows in both graphs mark 1/time to-peak AHP

(see Fig 5 A) It can however be noted that the 2nd interval in the neurone does not shorten to the same extent as in the model In fact when computing the successive f/i relations in the model limiting the shortening of the 2nd interval to lower frequencies than the 1st the jump tendency in the model firing is also decreasing

It has been reported that in some neurones the adaptation at the higher frequencies is followed by a *later increase in firing before the frequency is stabilized* (*Schwindt and Calvin* 1972) thus resembling the behaviour described above This adaptation acceleration sequence is however more longlasting than that of the model and that shown in Fig 7 and must then be related to another process than the $G_K(t)$ summation

It has previously been reported that the transition frequency from primary to secondary range is rather constant during a discharge (Kernell *1965 c) From the* model *firing it is predicted that the transition frequency* should increase during a discharge being *below the 1/time to-peak AHP for the 1st interval and* approaching that value for successive intervals Such an increase *in transition frequency can*

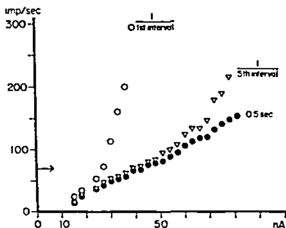


Fig 8 Frequency-current relation in lumbar motoneurone spike size 100 Same neurone as in Fig 1 A (Baldissera and Gustafsson 1974 a) In the graph are plotted the reciprocal of the 1st interval (open circles) 5th interval (open triangles) and the mean frequency measured at 0 5 s after the current onset (filled circles) on the ordinate against the current injected on the abscissa The arrow marks the 1/time to-peak AHP

also be found in real neurones and is best illustrated in Fig 7 B (see also Fig 7 A and Fig 8)

In the computed steady state f/i relation (Fig 6) a small deviation from linearity could be observed in the lowermost part of the curve Such a deviation could in fact also be observed in many neurones and is illustrated from one neurone in Fig 8 where the 1st and 5th interval f/i relations are shown together with the f/i relation measured 0 5 s after the current onset (mean of 4 successive intervals)

Trajectories The sequential changes in the trajectory of AHPs could also be identified and are illustrated from two motoneurones in Fig 9 In both these neurones the first interval trajectory assumes an upward convex (or hump-like) scoop shape already for low current intensities as earlier demonstrated both for motoneurones and the model (Baldissera and Gustafsson 1974 b) while the 2nd and 3rd interval for the same currents still keep the upward concave scoop shape Note however the development in these intervals of an upward convex (or hump-like) scoop with increasing current similar to the model behaviour In the computations this convexity decreased for the 2nd interval and got more pronounced for the 3rd interval when the first interval decreased rapidly in the $G_K(t)$ plateau region For the two neurones this decrease occurs at 22 nA (A) and 21 nA (B) (note the interval jump in A) and the trajectory change described above is easily noted With increasing currents the hump reappears for the 2nd interval (28 nA in A 29 and 31 nA in B) and gets more accentuated for the 3rd one (24—28 nA 31 nA upper record in B) When the 2nd interval decreases rapidly (note the jump at 31 nA in B) the 3rd interval assumes again a more rounded shape (31 nA lower record in B) and the 4th interval gets a more pronounced hump or upward convexity

I/i relations Besides the similarities between the model and the motoneuronal firing observed in the f/i plots when comparing the i/i/ relations in Fig 4 (model) and Fig 10 A and B (motoneurones) it can be noted that the shift to the right of

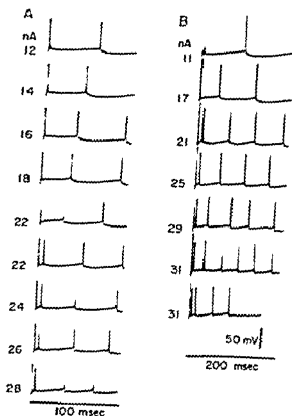


Fig 9

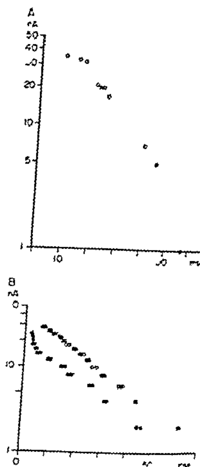


Fig 10

Fig 9 Trajectory modifications with increasing current for two lumbar motoneurones Spike sizes in A 83 mV and B 85 mV (see text)

Fig 10 Interval-current relation for two lumbar motoneurones A Same cell as in Fig 2 A and 7 A In the graph are plotted the 1st interval (filled circles) the 2nd interval (crosses) and the 3rd interval (open circles) on the abscissa against the current injected exceeding the rheobase on the ordinate (logarithmic) B The same plot as in A for the first two intervals in another motoneurone Same cell as in Fig 1 First interval filled circles and second interval (open circles)

the 2nd interval I/i relation with respect to the 1st interval is much more expressed in the motoneurones than in the model giving a more parallel relation between the I/i relations in motoneurones This behaviour was present in all the neurones examined An important factor for explaining this behaviour is that the threshold current for the third spike is usually slightly higher than that for the second spike (see Section VI Baldissera and Gustafsson 1974 b) a fact which will tend to shift the second interval I/i curve to the right since the plots are performed with a common threshold In fact because of the logarithmic ordinate a difference of

nA would shift the curves for the 2nd and 3rd interval a considerable amount in he lowermost part of the graph

In a preceding section describing the AHP summation it was noted that the ummation in many neurones deviated from the algebraical being both smaller and arger Due to the difficulties in evaluating the summation time course in single neurones a direct compari on between the amount of adaptation and the amount of AHP summation was only tried in a few cells in the present investigations In ection I the summation time course was described for two motoneurones one with approximate linear summation and one with considerable more than algebraical ummation In Fig 10 are shown the respective i/i relations for these two neurones Note that in the plateau region the adaptation in these two neurones was as expected by the summation of the respective AHPs The neurone in A is the same as that in Fig 2 A where the increase in computed AHP conductance was about twice that expected from an algebraical summation Correspondingly the current needed to bring the 2nd interval to the plateau region is 3 times that for the first interval In the econd neurone (Fig 10 B and Fig 1) the summation was close to algebraical and the corresponding current to bring the 2nd interval to the plateau is also twice that for the first interval

Discussion

In this paper ome experimental evidence was given of an approximate algebraical summation of the AHP conductance following repetitive spikes in motoneurones These results suggested that repetitive firing of motoneurones might be predicted as a first approximation by implementing a simple model (Baldissera and Gustafs on 1974 a b) with an algebraical summation of the AHP conductance changes A more comprehensive analysis of the AHP summation mechanisms will be dealt with in a forthcoming paper on DSCT neurones (Gustafsson *et al* unpubl) and the discussion will be postponed to that paper The discussion will instead be centred around the firing behaviour of the model compared to that of real moto neurones

From the result sections it is evident that the implemented model fits with many of the constraints imposed from the studies on repetitive firing in motoneurones The f/i relations of the model display the three firing ranges found in motoneurones (Kernell 1965 b Schwindt 1973) and the primary secondary range transition occurs close to the frequency corresponding to 1/time to peak AHP (Kernell 1965 c) The characteristic modifications in the AHP trajectories by increasing current described by Schwindt and Calvin (1972) and Schwindt (1973) are also found in the model firing Moreover the model did also forecast certain characteristics of the f/i relations and of the trajectories which were not earlier described and which were subsequently confirmed in real neurones such as the sequential changes in the f/i relations and in the trajectories during adaptation

The close similarity between the firing in the model and in real motoneurones

then justifies the conclusion that motoneuronal firing behaviour basically has to be related to the time course of the AHP conductance change as measured after single spikes Other factors have, however been suggested to participate in the firing regulation as discussed in the introduction of a previous paper (Baldissera and Gustafsson 1974 a)

The idea of a spatial factor intervening in the firing regulation, e g that a block in the dendritic invasion should cause the firing to change from the 'primary' to the secondary range was supported by the observation of a sudden change in AHP trajectory when entering the secondary range (Kernell 1965 b) and by the effect of synaptic currents on the f/i relations (Granit et al 1966 b) However it has been shown later that the trajectory change associated with secondary range firing is developing gradually (Schwindt and Calvin 1972) and that it is often found fully developed also in the primary range of firing especially for the first interspike interval (Fig 5 Baldissera and Gustafsson 1974 b) Moreover the effect of synaptic currents on the f/i relations and trajectories has recently been re-examined and no effects were found on either the f/i relation or the trajectories (Calvin and Schwindt 1973)

The suggestion forwarded by Schwindt and Calvin (1972) that the spatial factor more or less continuously affected the firing was based partly on theoretical considerations and partly on the suspicion that the trajectory modifications they described could not be envisaged by a model based on fixed postspike conductance changes as those forwarded in this paper The answer to this suggestion is given by the trajectories displayed by the model and no further comment is needed

In conclusion it seems that there is neither any evidence nor any need for postulating that a spatial factor should directly contribute to the motoneuronal firing behaviour as described by previous authors (Kernell 1965 b Schwindt and Calvin 1972 Schwindt 1973)

The descriptive terminology and the model properties

The descriptive terms for the firing curves and for the AHP trajectories used in this and in preceding papers have not been developed in relation to the AHP conductance mechanism and have often been attributed to other processes (Kernell 1965 b Schwindt and Calvin 1972 Schwindt 1973 It seems therefore important after the results reported here to discuss these descriptive terms in connexion with the AHP conductance changes

Terminology of the f/i relation

Primary range The primary range of firing was originally defined as an approximately linear f/i relation extending for each interval from the minimal frequency up to a frequency level at or somewhat below the 1/time to peak AHP (Kernell 1965 b c) However the analysis of the model behaviour and a careful evaluation of the firing curves obtained in the present experiments seem to indicate that the firing below 1/time to-peak AHP is nonlinear for all intervals and that it is regulated for

different intervals by different conductance time courses The f/i relation for the first interval is regulated by the exponential decay of the $G_K(t)$ and is therefore the lower arm of a continuous upward concave relation For the immediately following intervals the f/i relations are clearly nonlinear because of the sequential changes which are caused by the firing acceleration of the preceding interval given by the conductance plateau At steady state the f/i relation is quite linear but deviates downward for the lower frequencies In view of this complexity the paradigmatic approximation of the f/i relations at frequencies lower than 1/time to peak AHP to a linear primary range seems therefore restrictive and obscuring the correlations between firing and the underlying processes

Secondary range Firing within the secondary range was defined as discharges at frequencies higher than those compatible with the linear f/i slope of the primary range (Kernell 1965 b) Defined in this manner the secondary range will be produced in the model by two mechanisms First an upward deviation from the approximate linearity will be given for the first interspike intervals by the exponential decay of the $G_K(t)$ This initial secondary range will however be reached at higher frequencies for each successive interval and will not appear in the steady state firing (see Fig 6) Second the presence of a pronounced plateau in the $G_K(t)$ decay will for the steady state firing give an upward deviation from linearity at a frequency corresponding to the transition from the plateau phase to the exponential decay For the first interval this mechanism will be superimposed on the secondary range already given by the first mechanism The secondary range is then reached at lower frequencies for the first intervals than for the steady state a behaviour also found in real neurones (Fig 7 and 8 *cf* however Kernell 1965 c) This analysis shows then that there can be different types of secondary range given by different mechanism For the first intervals the detailed character of firing in this region is moreover strictly correlated with the shape of the $G_K(t)$ time course in the plateau region The presence of a pronounced local minimum in the $G_K(t)$ gives a jump in the firing from 1/time to peak AHP to 1/time to peak of the hump (see section VI b Baldissera and Gustafsson 1974 b), *i e* a jump from the upper limit of the primary to the lower limit of the tertiary range On the other extreme an exponential $G_K(t)$ decay gives a smooth concavity for the first intervals which progressively disappears for steady state firing (Kernell 1968 MacGregor and Sharpless 1973 see also Gustafsson *et al* 1972) Observe that this analysis is based upon the assumption of an algebraical AHP summation If the summation is not algebraical (see section I) additional mechanisms contributing to a secondary range of firing can also be considered (see below)

The term secondary range to denote deviations from linearity at higher frequencies seems then too unifying and the linear force fitting of the different f/i shapes displayed in this range tends as for the primary range to mask the characters of the underlying conductance processes

Trajectories In the analysis of motoneuronal firing behaviour based on the interspike voltage trajectories (Schwindt and Calvin 1972) the results were discussed in

terms of a separate 'early trajectory process or scoop controlling the firing rate and of a stereotyped late trajectory process - or ramp The increase in depolarizing current wa thought to modify the trajectory shape only in the scoop phase while the slope of the ramp remained constant (stereotyped)

In the model the scoop is given by the plateau phase of the $G_K(t)$ and the ramp by the subsequent exponential decay With increasing current there is in the model both a scoop decrease and a ramp slope change, i e there is no stereotyped ramp behaviour (Fig 3 C) Nevertheless the model behaviour is not in contrast to the experimental results by Schwindt and Calvin (1972) While they emphasi ed the stereotyped character of the ramp they were in fact able to force fit the ramps given by increasing currents throughout the primary range to successively steeper lines (see Schwindt and Calvin 1972) in much the same way as shown for the model in Fig 3 C where the same force fitting has been performed The same authors found also evident changes in the ramp slope at the longer intervals i e when the ramp changes in the model trajectories are most accentuated In conclusion because of the continuous change in the slope of the ramp portion of the AHP trajectory the term stereotyped does not seem the most proper one

It may be asked why the ramp slope in motoneurones is changing less than in other types of nerve cells (Schwindt and Calvin 1972) The reason for this behaviour lies in the fact that the point of origin of the ramp at the end of the scoop undergoes marked displacements with current injection If the ramp i e the $G_K(t)$ decay would start directly from the end of the falling phase of the spike where the conductance is high and the potential is not much affected by current injection then the ramp slope change would necessarily be much more marked The explanation is thus not that the underlying process of the ramp is separated from that of the scoop as earlier suggested (Schwindt and Calvin 1972) but instead that they are coupled

The trajectory analysis by Schwindt and Calvin (1972) suggested that firing within the primary range was associated with 1) a scoop change behaviour where the scoop decreased from the upward convex hape to a flattened shape and 2) a stereotyped ramp This trajectory behaviour was subsequently referred to as primary range trajectory behaviour From the above discussion it is clear that a prerequisite for an approximation to this behaviour is the pre ence of the $G_K(t)$ plateau In neurones lacking a $G_K(t)$ plateau no primary range trajectory behaviour will be present When considering this term it is of interest to note that such neurones (under the condition of algebraical summation) in the steady state will display *only* the primary range of firing

Later Schwindt (1973) recognized that in the secondary range there are changes in the rate of rise of the trajectory to firing level (as also found in the model see Fig 3) and discussed the general significance of this finding suggesting that such changes could influence the slope of the f/i relation in the presence of synaptic activity By calculations he found that the addition of synaptic current would not influence the f/i slope if the ramp behaviour is stereotyped i e the ramp slope is constant but would increase the f/i slope if the ramp slope is increasing with increasing current This effect would mean according to Schwindt (1973) that the f/i slope in motoneurones should be unaffected by synaptic activation during primary range firing as also found in real motoneurones (Granit *et al* 1966 a) but would be increased d ring secondary range firing a behaviour sometimes also noted by Granit *et al* (1966 b)

The result of the theoretical calculation is however surprising in view of other findings In the dorsal spinocerebellar tract neurones which according to Schwindt exhibit secondary

ange trajectory behaviour i e ramp slope changes the summation between injected and ynaptic current is linear i e there is no f/i slope change (Eide *et al* 1969) Recently Calvin nd Schwindt (1973) also found that the f/i slope in motoneurones in secondary range is iot affected by synaptic activation Moreover if the ramp slope is changing during primary ange in motoneurones as is shown by the model and as is also evident from published results Schwindt and Calvin 1972) the f/i slope would also be expected to change during primary ange firing

These discrepancies however are more apparent than real Schwindt s calculation in fact s based on the implicit assumptions 1) that the process underlying the ramp has similar properties regardless of the slope being changed (Schwindt 1973 Fig 5 A) or not (Fig 5 B) with injected current and 2) that the conductance is constant throughout the ramp The postulate of a stereotyped ramp (case B) obviously requires that the resistance must be con stant throughout the ramp The calculation based on th s assumption giving a f/i slope not affected by synaptic currents is thus correct

But in the case of a changing ramp slope the resistance must necessarily be smaller in the beginning of the ramp than later on This would mean that a calculation based on a fixed synaptic voltage produced by a certain synaptic current cannot be correct (see Fig 5 Schwindt 1973) If the calculation instead is based on the fact that R_m (input resistance) is changing during the ramp proportional to the interspike interval (R_m = / T see Fig 5) the solution of the equation ill give that the f/i slope is unaffected by synaptic activation also when the ramp slope is changing thus in line ith the experimental results mentioned above Theoreti cal objections suggesting that the changes in the ramp slope with injected current would affect the slope of the f/i relation during synaptic activation seem therefore immaterial

Schwindt and Calvin (1972) claimed that the entrance into the secondary range was a sociated with a flattened or inverted i e upward convex scoop shape and in some cells also by the appearance of a hump-type early potential similar to a hump type DD It was however noted that in some neurones the secondary range trajec tories were found also in steady state primary range firing and in the preceding article (Baldissera and Gustafsson 1974 b) we demonstrated that for the first inter val this trajectory shape usually is reached well within the primary range An ob ligatory coupling between trajectory shape and firing range seems thus not to exist This conclusion is further emphasized by a clear misconception also caused by this descriptive approach From published records from the DSCT neurones it was con cluded (Schwindt and Calvin 1972) that these neurones exhibited a trajectory shape comparable to that of motoneurones in secondary range in spite of a lack of secondary range of firing It is however perfectly clear that the upward con vexity in these neurones is given by the DD present also in resting conditions and is thus not comparable to the upward convexity in motoneurones given by the $G_K(t)$ plateau (for differences between the DD and the hump connected with the $G_K(t)$ time course see Baldissera and Gustafsson 1974 a b)

Tertiary range The tertiary range of firing was defined for the steady state firing as a 3rd linear tract in the f/i relation following the secondary range (Schwindt 1973) This deviation appeared to be associated with a sudden change in the trajectory shape which was interpreted as indicative of a change in the cell properties e g of a sudden decrease in antidromic invasion or of the drop out of a conductance component In the tertiary range the trajectory shape (Schwindt 1973) was angular with a downward peak Increasing currents modified the trajec tory by decreasing the peak leaving the slope of the rising phase constant i e in a manner analogous to the so called primary range trajectory behaviour In the mo del this deviation from the secondary range slope is also occurring at the h

frequencies and is associated with a drop out of a conductance component namely the $G_K(t)$ plateau phase This behaviour is most dramatically illustrated by the jump present in both the model firing and in real motoneurones (Baldissera and Gustafsson 1974 b) Throughout this jump the upward convexity in the trajectory given by the plateau is bypassed and the trajectory assumes the angular form However, since in the model the beginning of the AHP is fixed to a certain potential (see Baldissera and Gustafsson 1974 b) the trajectory modification in 'tertiary range will necessarily be mainly of the ramp slope change type and not of the scoop change type described for real motoneurones

The description of a new linear range of firing as made by Schwindt (1973) seems an attempt to overemphasize the well known and expected approach of firing to the upper limit fixed by the absolute refractoriness of the spike This general behaviour has been already described in motoneurones (Granit *et al* 1963) and in many other cells *e g* the eccentric cells of Limulus (Fuortes and Mantigazzini 1962) the pyramidal tract cells (Koike *et al* 1970) the trochlear motoneurones (Baker and Precht 1972), and has also been deduced from Hodgkin Huxley equations for the squid giant axon (Agin 1964)

The proofs for the existence of a second less frequent type of tertiary range with a higher slope than the secondary' (Schwindt 1973) seem contradictory to the description the same author gave for the tertiary' range trajectory In that paper Fig 1 A the nonlinear f/i relation above 1/time to-peak AHP was divided into two linear ranges From the trajectories in the corresponding records in Fig 1 B—E this part of the curve looks related with the transition of the firing point over the plateau region and the angular trajectory shape indicative of the entrance in the tertiary' range is only reached at the points 6 and 7 concomitantly with the initial downward bending of the f/i relation *i e* with the entrance into the true tertiary range The concave upward f/i relation above 1/time to peak AHP would then be better described in usual terms as a secondary range which as previously discussed *is not* necessarily approximated by a line

Limitations of the model

The present model does not take into account all the various factors influencing the f/i relations and the trajectories a series of which will be shortly *considered*

The presence of a well developed DD gives rise to much more complicated trajectories especially in their early part

The difference between the rheobasic current and the current needed *to evoke* rhythmic activity (Kernell 1965 a) seems to indicate a possible *role of accomodation* in firing regulation This threshold difference is however most likely caused by a current threshold change given by the sag process not included in the present computations than to a real voltage threshold change (Baldissera and Gustafsson 1974 b) This suggestion would then be in line with the postulate *that motoneurones* responding with a sustained repetitive discharge to depolarizing *current* injection do not show any evident accomodation to ramp currents (*Burke* and Nelson 1971)

In the model the adaptation expressed as changes in the f/i slope will be over within the two first intervals in the primary range Even if the adaptation *in real* neurones is largely over within the same intervals there is in motoneurones a further

decrease in f/i slope which may continue for as long as 1 second (Kernell 1965 a) that must be caused by another process

In the steady state firing the secondary range should not be reached by the model until the frequency exceeds that corresponding to the end of the plateau phase (or approximately the 1/time to-peak AHP) It was however reported that the transition frequency also for steady state firing can be appreciably lower than this limiting frequency (Kernell 1965 c) Moreover the ratio between the second ary / primary f/i slopes for the steady state firing can be appreciably higher in real neurones (Kernell 1965 b) than that given by the model A possible explana tion for these discrepancies might be found in the fact that the AHP summation in real neurones may deviate from that of the model *i e* from the algebraical In fact if the correction factor (see section I) is not constant but decreases with decreasing interval (see Fig 2 C) *i e* the conductance summation goes against a limiting value less than the algebraical the secondary range would be expected to start at lower frequencies and deviate more from the primary range f/i slope than that predict ed by the model Such a change in the correction factor could then be an additio-nal (the 3rd) mechanism to the other two previously mentioned in the discussion giving rise to a secondary range of firing It should then be remembered that such a mechanism would not only accentuate the steady state secondary range given by the $G_K(t)$ plateau but would also give a steady state secondary range in a model based on a pure exponential decay The recent suggestion that the simple model of Kernell (1968) based on an exponential $G_K(t)$ decay would not be able to give a secondary range in steady state (MacGregor and Sharpless 1973) due to the AHP summation (which was without saying equated to algebraical summation) seems then not warranted in view of the deviations from algebraical summation already encountered between the first two spikes in real motoneurones (see section I)

In motoneurones with a less pronounced $G_K(t)$ plateau the firing would be more similar to that of the model version lacking plateau i e the steady state secondary range could even be (if algebraical summation) hardly present in such cells This prediction fits then well with the recent observation (Schwindt 1973) that motoneurones lacking steady state secondary range even up to very high dis charge frequencies have a shorter AHP duration and thus likely a shorter time to-peak AHP (Kernell 1965 c) and display more adaption than neurones giving steady state secondary range

In the model there is no frequency limiting process *i e* steady state frequencies up to several hundred imp/s can be reached and thus also in steady state firing all the firing ranges will be present In real motoneurones however the steady firing gets irregular and finally stops in many neurones even before having reached the secondary range and for any firing range frequencies above 150—200 imp/s are never found for steady firing (Kernell 1965 b) A likely explanation for the lack of high frequency firing is a gradual development of sodium conductance inac tivation as indicated by the decrease in spike height and the broadening of the spikes observed to develop gradually during secondary range firing together with an increase in firing level (Schwindt 1973)

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:RKE R. E. and P G NELSON Accomodation to current ramps in motoneurons of fast and low twitch motor units *Intern J Neuroscience* 1971 *1* 347—356
ALVIN W H and P SCHWINDT Steps in production of motoneuron pikes during rhythmic firing *J Neurophysiol* 1972 *35* 297—310
DE E L FEDINA J JANSEN A LUNDBERG and L VYKLICKY Properties of Clarke s column neurones *Acta physiol scand* 1969 *77* 125—144
CORTES M G F and F MANTEGAZZINI Interpretation of the repetitive firing of nerve cells *J gen Physiol* 1962 *45* 1163—1179
RANIT R. D KERNELL and G K. SHORTESS Quantitative aspects of repetitive firing of mammalian motoneurones caused by injected currents *J Physiol* (Lond) 1963 *169* 911—931
GRANIT R D KERNELL and Y LAMARRE Algebraical summation in synaptic activation of motoneurones firing within the primary range to injected currents *J Physiol* (Lond) 1966 a *187* 379—399
GRANIT R. D KERNELL and Y LAMARRE Synaptic stimulation superimposed on motoneurones firing in the secondary range to injected current *J Physiol* (Lond) 1966 b *187* 401—415
GUSTAFSSON B S LINDSTROM and M TAKATA Repetitive firing in dorsal spinocerebellar tract neurones *Brain Res* 1972 *47* 506—509
GUSTAFSSON B and P ZANGGER, "Depression of the afterhyperpolarization in dorsal spinocerebellar tract neurones *Brain Res* 1974 *72* 320—322
ITO M and O HIMA, Temporal summation of after hyperpolarization following a motoneuron spike *Nature* (Lond) 1962 *195* 910—911
KERNELL D The adaptation and the relation between discharge frequency and current strength of cat lumbosacral motoneurones stimulated by long lasting injected currents *Acta physiol scand* 1965 a *65* 65—73
KERNELL D High frequency repetitive firing of cat lumbosacral motoneurones stimulated by long lasting injected currents *Acta physiol scand* 1965 b *65* 74—86
KERNELL, D The limits of firing frequency in cat lumbosacral motoneurones possessing different time course of afterhyperpolarization *Acta physiol scand* 1965 c *65* 87—100
KERNELL D Repetitive impulse discharge of a imple neurone model compared to that of spinal motoneurones *Brain Res* 1968 *11* 685—687
KERNELL D and H SJÖHOLM Repetitive impulse firing Comparisons between neurone models based on "voltage clamp equations" and spinal motoneurones *Acta physiol scand* 1973 *87* 40—56
KOIKE H Y MANO Y OKADA and T OHIMA Repetitive impulses generated in fast and slow pyramidal tract cells by intracellularly applied current steps *Exp Brain Res* 1970 *11* 263—281
KUNO M J T MIYAHARA and J N WEAKLEY Post tetanic hyperpolarization produced by an electrogenic pump in dorsal spinocerebellar tract neurones of the cat *J Physiol* (Lond) *210* 839—855
MAC GREGOR R. J and S K. SHARPLESS Repetitive discharge rate of a simple neuron model with accumulation of afterhyperpolarization conductance *Brain Res* 1973 *64* 387—390
SCHWINDT P C Membrane potential trajectories underlying motoneuron rhythmic firing at high rates *J Neurophysiol* 1973 *36* 434—449
SCHWINDT P C and W H CALVIN Membrane potential trajectories between spikes underlying motoneuron firing rates *J Neurophysiol* 1972 *35* 311—325
SCHWINDT P C and W H. CALVIN Equivalence of synaptic and injected current in determining the membrane potential trajectory during motoneuron rhythmic firing *Brain Res* 1973 *59* 389—394
TAKAHASHI K Slow and fast groups of pyramidal tract cells and their respective membrane properties *J Neurophysiol* 1965 *28* 908—924

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From the Departments of Pharmacology and Endodontics Karolinska Institutet Stockholm Sweden

Excitation of Intradental Sensory Units by Pharmacological Agents

By

Leif Olgart

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Abstract

Olgart L *Excitation of intradental sensory units by pharmacological agents* Acta physiol scand *1974* *92* 48—55

Recordings were made from intradental sensory nerves in the tooth of the cat Local application of bradykinin and histamine failed to induce impulse activity even when applied to the exposed pulp whereas 5 hydroxytryptamine produced *bursts of impulses with short* latency lasting a few seconds Application of compound 48/80 induced an increase *in* impulse activity after a delay of some minutes and this activity persisted for about 2 *h irrespective* of whether the agent was removed or not The impulse frequency decreased promptly when *the temperature* was *raised* and *recovered* when *the* temperature was *lowered again* Pre treatment with polyphloretin phosphate (PPP) inhibited the excitatory action of compound 48/80 The threshold of excitation to cold stimulation was lowered after introduction of compound 48/80 Since the principal action of compound 48/80 is to release endogenous *substances from mast cells the present findings may indicate that the increase in impulse* activity is secondary to a release of biogenic substances However at present a direct *action* of compound 48/80 can not be excluded

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Biogenic substances such as bradykinin and 5 hydroxytryptamine (5 HT) have *been shown to cause pain in the human* blister base (Armstrong *et al* 1953) while histamine produces essentially an intense itching sensation (Keele and Armstrong 1968) Dellow and Roberts (1966) reported that no pain *sensation* was obtained when bradykinin was applied to exposed human dentin and Kroeger *and* Krivoy (1963) found that bradykinin did not excite intradental nerves *in rats* and dogs On the other hand Anderson and Naylor (1962) showed that histamine causes pain when applied to the human dental pulp

The *present* investigation was conducted to elucidate the excitatory *action* of substances which may be released endogenously and which may *influence* the excitability of sensory units in the tooth

Furthermore the nerve response to compound 48/80 an agent which *is* known to release mast cell *bound biogenic substances has been studied*

Methods

Adult cats (2—3 kg and 1—3 years old) were anesthetized with sodium pentobarbital (30 mg/kg i.v.) or chloralose (40 mg/kg) and urethane (50 mg/kg). The experimental procedure used was similar to that described by Olgart, Haegerstam and Edwall (1974).

Two cavities were prepared in the canine teeth, one over the pulp horn and the other within the gingival part of the crown, leaving 0.1—0.3 mm of intact dentin over the pulp. The cavities were filled with isotonic saline solution and Plastibase (Squibb) insulating gel was applied around the cavities. Platinum recording electrodes were placed in contact with the exposed dentin and recordings from intradental sensory nerve units were made from the coronal cavity using the technique described by Edwall and Scott (1971).

Since no spontaneous activity was usually recorded, the responsiveness of units located in the vicinity of the coronal cavity was tested by applying hypertonic sodium chloride (1.54 M). The latent period of the resulting activity was noted and the cavity was deepened until a latency of about 40 s was obtained. This parameter was used to obtain standard preparations. The activity was rapidly abolished after washing the cavity with isotonic saline solution. In some experiments the hypertonic solution was also used to test the excitability after the various experimental procedures (Olgart, unpublished results).

The temperature of the tooth could be kept constant or could be rapidly changed by means of a water circulated thermode in contact with the tooth crown. The temperature was continuously monitored using thermocouples in contact with the tooth surface and the thermode close to the recording cavity. Control temperature was 32° C.

Agents were introduced in the coronal cavity in a volume of 0.2 μl after removal of the saline solution. To prevent evaporation during long procedures a thin plastic film was placed over the cavity.

Material

Histamine (10 mg/ml), bradykinin (10 mg/ml), 5-hydroxytryptamine (5-HT) (1 mg/ml), acetylcholine (10 mg/ml), Compound 48/80 (0.5 mg/ml, 1 mg/ml), polyphloretin phosphate (PPP) (1 mg/ml, 10 mg/ml, 100 mg/ml), calcium chloride (18 mM).

Compound 48/80 and PPP was generously supplied by Dr B. Fredholm, AB Leo, Helsingborg, Sweden. The other substances used were obtained from normal commercial sources. The solutions were made isotonic when possible.

Results

Application of 5-hydroxytryptamine (5-HT), bradykinin and histamine. Both bradykinin (10 mg/ml) and histamine (10 mg/ml) failed to excite the intradental sensory neurons. This was a consistent finding in cases with cavities of standard depth devoid of spontaneous activity (18 procedures in 6 animals) as well as in deeper cavities with pulp exposure showing spontaneous activity (8 procedures in 4 animals).

5-HT (1 mg/ml) caused an immediate increase of the impulse activity (usually within 1 s) which lasted for about 5—10 s (10 procedures in 6 animals). The nerve units were insensitive to a second application of 5-HT during the 10 minutes after a previous response to 5-HT.

Application of compound 48/80. When compound 48/80 was introduced in the recording cavity, nerve impulse activity started after a latency of about 1 min. There was always a latent period irrespective of whether the pulp tissue was covered with intact dentin or was exposed. The impulse frequency reached a steady state level of 100—200 imp/s within one minute. Removal of compound 48/80 by absorbent paper and subsequent repeated washing with isotonic saline did not influence the activity. However, there was a gradual decrease in activity, which

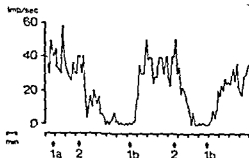


Fig 1 The effect of isotonic sodium chloride and calcium chloride on the sensory unit impulse frequency elicited by compound 48/80 (1 mg/ml) Local application in the recording cavity Chloralose — urethane 1 a, b Sodium chloride isotonic 2 Calcium chloride 18 mM Impulse frequency represents the running average for 10 s interval and is given as imp/s

reached control level within 120 min Fig 1 shows the impulse activity 10 min after the application of compound 48/80 (1 mg/ml) The compound had been removed from the cavity one min after the activity had started This procedure was followed by several washings one of which is shown in the figure (Fig 1 1 a) The washings had no obvious influence on the activity However when the saline olution was replaced by calcium chloride (18 mM) (Fig 1 2) the impulse activity was abol shed Removal of calcium chloride and subsequent washing with saline rapidly restored the activity (Fig 1 1 b) As can be seen this procedure could be repeated with essentially the same result This experiment repre ents the typical pattern of response obtained in 10 procedures in 3 animals

Effect of temperature In order to further analyse the action of compound 48/80 the effect of raised tooth temperature was investigated In Fig 2 a typical recording of the effect of increased tooth temperature on the activity to compound 48/80 is shown Application of compound 48/80 at a temperature of 50 C increased the impulse frequency after a latent period of 10 min When the temperature was lowered to 32° C the frequency increased rapidly A subsequent increase in the temperature to 50 C produced a reduction in the frequency which lasted as long as the temperature remained elevated This pattern of response was obtained in 17 procedures in 4 animals The inhibitory effect of heat was a consistent finding when compound 48/80 was present in the cavity as well as when the agent had been re placed by isotonic saline Fig 3 shows a typical experiment in which heat was applied 70 min after the removal of compound 48/80 from the cavity Isoto c saline was present in the cavity during the increase in temperature As can be seen the impulse frequency showed a marked decrease when the temperature was raised to 50 C and recovered when the temperature was lowered to 32 C

Influence of polyphyloretin phosphate (PPP) on the nervous response to compound 48/80 In 9 procedures in 3 animals PPP (10 mg/ml) was introduced into the recording cavity for 15 min The cavity was thereafter washed repeatedly and filled with isotonic saline for 5 min before compound 48/80 was applied It was a consistent finding that compound 48/80 (1 mg/ml) failed to induce any impulse activity within one hour after application of PPP PPP (1 mg/ml) inhibited the effect of compound 48/80 (0 5 mg/ml) whereas the excitatory action of acetyl

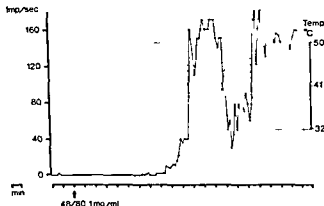


Fig 2 Influence of increased tooth temperature (— — —) on the sensory unit impulse frequency (——) elicited by compound 48/80 Chloralose — urethane

choline (10 mg/ml) and 5 HT (1 mg/ml) was not inhibited (3 procedures 2 animals) In 4 procedures (3 animals) using PPP (100 mg/ml) a subsequent introduction of hypertonic sodium chloride (1 54 M) induced the same pattern of response as was obtained before the application of PPP into the cavity

Cold stimulation after application of compound 48/80 Before application of compound 48/80 when the cavities were filled with isotonic saline solution a rapid decrease in temperature from 34° C to 27° C failed to excite the sensory neurons However after application of compound 48/80 (1 mg/ml) similar cold stimulation consistently caused a transient increase in the impulse frequency A typical result is shown in Fig 4 This pattern of response was obtained in 12 procedures in 4 animals

Discussion

This investigation has shown that intradental sensory nerves are not excited by bradykinin or histamine while 5 HT and compound 48/80 induce an impulse activity with different patterns of response 5 HT causes an immediate burst of



Fig 3 Influence of increased tooth temperature (— — —) on the impulse frequency (——) elicited by compound 48/80 (1 mg/ml) Recording started 10 min after removal of compound 48/80 The cavity was filled with isotonic sodium chloride Chloralose — urethane

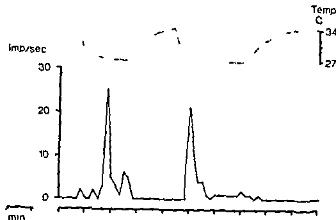


Fig 4 The effect of cold stimulations on impulse frequency 10½ min after local application of compound 48/80 (1 mg/ml) The recording cavity was filled with isotonic sodium chloride Temperature (— — —) Impulse frequency (——) Chloralose — urethane

impulses lasting 5—10 s whereas compound 48/80 produces impulse activity after a latency of about 4 min which lasts for about 2 h

The inability of bradykinin to excite intradental sensory neurons when applied to exposed pulp tissue was previously reported by Kroeger and Krivoy (1963) who recorded activity from the inferior alveolar nerve in rats and dogs Experiments in human subjects (Dellow and Roberts 1966) have shown that bradykinin applied to exposed dentin failed to cause pain Apart from these negative findings including the present results it is well known from studies on other tissues that bradykinin is a potent pain stimulating substance This has been shown using the blister base technique on human skin (Keele and Armstrong 1964) and by intraarterial or intraperitoneal injection in the dog (Guzman Braun and Lim [illegible] Dickersson et al [illegible]) and in man (Sicuteri Fanciullacci and Anselmi 1963 Lim et al 1966) Thus there seems to be a difference in sensitivity to bradykinin between the intradental sensory neurons and dermal and visceral sensory neurons The difference may be related to the existence of paravascular chemosensitive pain receptors in dermal and visceral tissues (Lim et al 1962 Guzman Braun and Lim 1962) which may not be present in the tooth This difference may also explain the inability of histamine to excite the intradental units in the cat

Both bradykinin and histamine evoke pain and vasomotor responses simultaneously when injected into dermal veins (Sicuteri 1968) and may thus indirectly influence the nerve response Changes in blood flow were shown to modulate the sensory nerve excitability in the cat tooth (Edwall and Scott 1971) Such influence seems however less probable in the present experiments since it was recently shown under the same experimental conditions that bradykinin and histamin did not change pulpal blood flow when injected intra arterially (Edwall Olgart and Haegerstam 1973)

5 HT evoked bursts of impulses with the same pattern of response as was obtained after application of acetylcholine (Edwall Haegerstam and Olgart 1974) 5 HT has been shown to cause pain in the human tooth (Anderson and Naylor 1962) and

in the blister base (Armstrong *et al* 1953) The present finding that 5 HT excited intradental sensory units in the cat may thus suggest that 5 HT bradykinin and histamine exert different actions on nerves

The delayed response after application of compound 48/80 and the long duration of the nerve activity were quite different from the response to other excitants previously tested in this preparation Thus acetylcholine and potassium chloride like 5 HT in the present study, were shown to excite the neurons within a second producing a transient impulse activity (Edwall Haegerstam and Olgart 1974, Arwill *et al* 1973) The delayed response to compound 48/80 in cavities with dentin covering the pulp as well as in cavities with a pulp exposure may be explained by a slow penetration of the compound and/or that the reactions induced by it proceed slowly Compound 48/80 has earlier been tested in the blister base in man and was shown to cause pain and itch with a latency of 1—2 min the effect having a duration of more than 10 min (Armstrong *et al* 1953) The mechanism of this action was however not analyzed

One possible action of compound 48/80 in the present study is a direct effect on the sensory nerve endings The substance which is a positively charged polymer amine may modify the properties of the nerve membrane leading to repetetive discharges in the fibres and may also change the excitability to other stimuli Heat reduced or abolished the excitation induced by compound 48/80 in contrast to the increase in impulse frequency produced by heat during excitation by low extracellular calcium and hypertonic salt solutions in the same experimental model (Olgart Haegerstam and Edwall 1974 Olgart unpublished results) Thus if the effect is a direct one compound 48/80 seems to act in a different manner than low extracellular ionized calcium and hypertonic salt solutions

The other possible action of compound 48/80 may be an indirect one The principal action of compound 48/80 is to release histamine by degranulating mast cells (Hogberg and Uvnas 1958 Chakravarty Hogberg and Uvnäs 1959) Furthermore the formation of biologically active principles including slow reacting substance (SRS) and pro taglandins seems to be related to the histamine release process induced by compound 48/80 as shown in the cat paw (Strandberg 1971 a) The inhibitory effect of heat upon the impulse activity in the present study is interesting in view of the observation by Strandberg (1971 b) that the yields of histamine and SRS in effluents from cat paws were decreased following temperature elevations Since histamine consistently failed to excite the intradental sensory nerves when applied locally histamine is probably not involved in the excitation elicited by compound 48/80 in the pulp

The inhibitory action of PPP upon the effect of compound 48/80 may be correlated with the finding of Mathe and Strandberg (1971) and Strandberg (1973) showing an inhibition of histamine release and SRS formation However the present finding may also be explained by a chemical interaction between PPP and compound 48/80 since PPP is acidic in nature and compound 48/80 is a basic polymer (*cf* Strandberg 1973)

So far no information is available concerning the existence of mast cells in the dental pulp of the cat Mast cells have been found in the normal dental pulp of the dog (Sulzmann 1966) and in the inflamed human pulp (Zachrisson and Skogedal 1971)

The question as to whether SRS excites nerve membranes remains to be elucidated On the other hand the pain producing activity of prostaglandins has been intensively studied Ferreira (1972) and Ferreira *et al* (1973) suggested that the main feature of their action was to produce hyperalgesia to mechanical chemical and other stimuli In view of these findings the raised excitability to cold stimulation found in the present study after application of compound 48/80 is interesting and may indicate that biologically active principles are involved in the compound 48/80 induced nerve activity The present findings indicate that further research on the mechanism involved in the compound 48/80 induced excitation of intradental sensory units is called for

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References

ANDERSON D J and M N NAYLOR Chemical excitants of pain in human dentine and dental pulp *Arch oral Biol* 1962 7 413—415

ARMSTRONG D R M L DRY C A KEELE and J W MARKHAM Observations on chemical excitants of cutaneous pain in man *J Physiol* (Lond) 1953 120 326—351

ARWILL T I EDWALL J LILJA L OLGART and S E SVENSSON Ultrastructure of nerves in the dentinal pulp border zone after sensory and autonomic nerve transection in the cat *Acta odont scand* 1973 *31* 273—281

CHAKRAVARTY N B HOGBERG and B UVNAS Mechanism of the release by compound 48/80 of histamine and of a lipid soluable smooth muscle stimulating principle (SRS) *Acta physiol scand* 1959 *45* 255—270

DELLOW P G and M L ROBERTS Bradykinin application to dentine A study of a sensory receptor mechanism *Aust dent J* 1966 *11* 384—387

DICKERSSON G D R J ENGLE F GUZMAN D W RODGERS and R K S LIM The intraperitoneal bradykinin evoked pain test for analgesia *Life Sci* 1965 *4* 2063—2069

EDWALL L and D SCOTT JR Influence of changes in microcirculation on the excitability of the sensory unit in the tooth of the cat *Acta physiol scand* 1971 *82* 555—566

EDWALL L L OLGART and G HAEGERSTAM Influence of vasodilator substances on pulpal blood flow in the cat *Acta odont scand* 1973 *31* 288—298

EDWALL L G HAEGERSTAM and L OLGART The excitatory action of acetylcholine on intradental sensory units *Acta physiol scand* 1974 In press

FERREIRA S H Prostaglandins aspirin like drugs and analgesia *Nature New Biol* 1972 *240* 200—203

FERREIRA S H S MONCADA and J R VANE Prostaglandins and the mechanism of analgesia produced by aspirin like drugs *Brit J Pharmac* 1973 *49* 86—97

GUZMAN F C BRAUN and R K S LIM Visceral pain and the pseudo affective response to intra arterial injection of bradykinin and other algesic agents *Arch int Pharmacodyn* 1962 *136* 353—384

HOGBERG B and B UVNAS Inhibitory action of allicin on degranulation of mast cells produced by compound 48/80 histamine liberator from ascaris lecithinase A and antigen *Acta physiol scand* 1958 *44* 157—162

KEELE C A and D ARMSTRONG *Substances producing pain and itch* Arnold London 1964

KEELE C A and D ARMSTRONG Mediators of pain In *Pharmacology of Pain* edited by R K S Lim Oxford Pergamon 1968 9 3—24

KROEGER D C and W A KRIVOY In Structure and function of biologically active peptides discussion following F W Horton The role of bradykinin in the peripheral nervous system *Ann N Y Acad Sci* 1963 *104* p 236

LIM R K S C N LIU F GUZMAN and C BRAUN Visceral receptors concerned in visceral pain and the pseudoaffective response to intra arterial injection of bradykinin and other algesic agents *J comp Neurol* 1962 *118* 269—277

LIM R K S D G MILLER F GUZMAN D W RODGERS S K WANG P Y CHAO and T Y SHIH *Relief of pain Site of action of narcotic and non narcotic analgesics* Scientific exhibit 115th Annual Convention Am med Ass Chicago June 26—30 1966

MATHE A A and K STRANDBERG Antagonism of slow reacting substance by polyphloretin phosphate on isolated human bronchi *Acta physiol scand* 1971 *82* 460—465

OLGART L G HAEGERSTAM and L EDWALL The effect of extracellular calcium on thermal excitability of the sensory unit in the cat *Acta physiol scand* 1974 *91* 116—122

SICUTERI F M FANCIULLACCI and B ANSELMI Bradykinin release and inactivation in man *Int Arch Allergy* 1963 *22* 77—84

SICUTERI F Sensitization of nocireceptors by 5 hydroxitryptamine in man In *Pharmacology of Pain* edited by R K S Lim Oxford Pergamon 1968 *9* 57—86

STRANDBERG K Efflux of slow reacting substance (SRS) and prostaglandins from cat paws perfused with d tubocurarine and n octylamine *Acta physiol scand* 1971 a, *82* 509—520

STRANDBERG K Release of histamine and formation of slow reacting substance in the cat paw induced by compound 48/80 *Acta physiol scand* 1971 b *82* 500—511

STRANDBERG K Inhibition of histamine release and formation of slow reacting substance by polyphloretin phosphate *Acta pharmacol* (Kbh) 1973 *32* 33—45

SULZMANN R Beitrage zur Histologie der Zahnpulpa II Licht und elektronenmikroskopische Darstellung von Plasmazellen und Gewebsmastzellen in permanenten monoradikularen Hund zahnen *Anat An* 1966 *19* 202—208

ZACHRISSON B U and O SKOGEDAL Mast cells in inflamed human dental pulp *Scand J dent Res* 1971 *79* 488—492

Acta physiol scand 1974 92 56—65

From the Department of Anatomy University of Helsinki, Finland

Incorporation of Imipramine into Isolated Rat Peritoneal Mast Cells *in Vitro*

By

J GRIPENBERG

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Abstract

GRIPENBERG J *Incorporation of imipramine into isolated rat peritoneal mast cells in vitro* Acta physiol scand 1974 92 56—65

The incorporation of imipramine into isolated rat peritoneal mast cells was fluorometrically studied *in vitro* The binding of imipramine was characterized by the following features 1 it took place *in two phases* the first of which was very rapid, 2 it increased *with increasing* exogenous concentration in a sigmoidal fashion 3 it increased with increasing exogenous pH 4 it was dependent on neither temperature nor calcium ions in the medium nor did the binding occur specifically to mast cells but also to leucocytes 5 it was reduced by several imipramine derivatives tricyclic antidepressants and 5-hydroxytryptamine at high concentrations 6 about 40 % of the cell bound drug was easily washed out the remaining 60 % represented a level approached after prolonged washing and seemed to be fairly tightly bound 7 the subcellular location of imipramine in mast cells was different from that of 5-hydroxytryptamine indicating a nongranular binding of the drug It is concluded that imipramine binds to mast cells in a passive manner and is associated with the *cell membrane* where it seems to be bound to at least 2 different sites At low concentrations imipramine is probably bound *to sites specifically influencing the* operativeness of the amine *carrier* Higher concentrations of imipramine results in binding to nonspecific sites resulting in impairment of the structure and function of the cell membrane

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Imipramine and related tricyclic antidepressant drugs are considered to exert their effects on the nervous system at least partially by inhibiting the transport of monoamines through the cell membrane This concerns the uptake of 5-hydroxytryptamine 5-HT in nervous tissue (*Carlsson et al* 1968 Carlsson 1970 Shaskan and Snyder 1970 as well as in several models for the monoaminergic nerve terminal including blood platelets Stacey 1961 Yates *et al* 1964 Ahtee *et al* 1968 Todrick and Tait 1969 Ahtee and Saarnivaara 1971) and mast cells (Jansson 1970 Fredholm 1972 In blood platelets the site of action of imipramine is apparently at the level of the cell membrane (Ahtee *et al* 1968 Da Prada and Pletscher 1968) This seems to be the case in mast cells as well as imipramine is known not to affect 5 HT kinetics in isolated membrane free mast cell granules (Jansson 1971) In contrast to imipramine reserpine and guanethidine readily interfere with the

uptake of 5 HT by isolated mast cell granules (Jansson 1971) This effect was explained by the observation that both reserpine and guanethidine became incorporated into the granules (Gripenberg *et al* 1972 Gripenberg 1973) As imipramine may bring about its effects on amine uptake and release mechanisms either *via* a specific effect on an amine carrier site or indirectly by impairing the functional integrity of the cell membrane a study on the incorporation of imipramine into mast cells seemed motivated Results to be presented indicate that imipramine may initially combine to specific sites blocking the amine carrier mechanism while at high concentrations the structure and function of the cell membrane are further impaired

Material and methods

Adult albino rats of both sexes of the Sprague Dawley strain were used The mixed pleural and peritoneal cells were washed out from the pleural and peritoneal cavities with 10 ml of incubation medium respectively

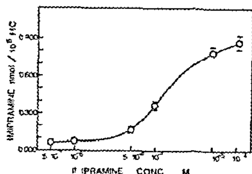
Isolation of mast cells and mast cell granules Isolation of mast cells was carried out by density gradient centrifugation according to Uvnas and Thon (1959) as described earlier (Gripenberg *et al* 1972) The gradient was composed of one layer of a 30 % solution of Ficoll® (Pharmacia) in saline supplemented with bovine serum albumine and glucose each at 0 1 % Mast cell granules were isolated as described by Lagunoff *et al* (1964) and by Thon and Uvnas (1966) by differential centrifugation in 0 32 M sucrose pH 6 9 after freezing and thawing of an isolated mast cell population

Incubation technique The cells were incubated in a Tris HCl buffered electrolyte solution the composition of which has been given earlier (Gripenberg 1973) The Tris HCl buffer routinely used was substituted by a sodium phosphate buffer at an equimolar concentration in the pH range 6 0—7 2 Incubations were carried out in siliconized glass tubes in a water bath at 37 C unless otherwise stated The incubation volume was 1 0—10 0 ml with an average cell concentration of $1\,0 \times 10^6$ mast cells per incubation After incubation the cells were diluted with ice chilled medium spun down at $800 \times g$ for 3 min and washed with 2 changes of fresh medium Mast cell granules were incubated in 0 32 M sucrose pH 6 9 at 23 C spun down at $2700 \times g$ for 30 min and washed with 2 changes of sucrose Mast cell and granule pellets were resuspended in 0 01 N HCl and stored at −35 C for not more than 3 days prior to determination of the imipramine and 5 HT contents The effect of various drugs on the binding of imipramine to mast cells was studied by the addition of drugs 30 min prior to the addition of imipramine All glassware including pipettes was siliconized (Siliclad® Clay Adams Co)

Determination of imipramine Imipramine was determined spectrophotofluorometrically according to a modification of a method described by Dingel *et al* (1964) The final sample volume was 90 μl Fluorescence was read at 415 nm after activation at 295 nm in an Aminco-Bowman spectrophotofluorometer The linearity of the method was extended down to 0 10 nmol/ml by the present semi micro modification The recovery of the method was good 96 4±3 1 (mean±S E) of the theoretical amount was recovered from internal standards None of the drugs used including imipramine derivatives interfered with the method at the concentrations used The amounts of imipramine reported were corrected for blanc values

Determination of 5 HT The 5 HT content of the cellular material was determined spectrophotofluorometrically using the method of Vanable (1963) after extraction of the amine as described by Snyder *et al* (1965) 5 Hydroxytryptamine creatinine sulphate was used as standard

Reagents and drugs Ultrapure water (Millipore®) was used throughout All reagents used were commercially available analytical grade products Both n butanol and n heptane were washed according to Weissbach (1961) For the extraction of imipramine iso-amyl alcohol was added to heptane at 1 5 % A 0 1 M solution of ninhydrin (Merck AG) was made up freshly for every determination of 5 HT Reagents Tris 7—9® (tris(hydroxymethyl)amino methane) EGTA (ethyleneglycol bis(amino-ethyl ether) N N tetra acetic acid) (Sigma) Drugs Imipramine HCl desmethylimipramine HCl 2 hydroxy imipramine HCl 2 hydroxy-desmethyl imipramine oxalate and chlorimipramine HCl were kindly donated by Ciba-Geigy AG Basel Switzerland 5 hydroxytryptamine creatinine sulphate and 5 hydroxytryptophan (Fluka AG)



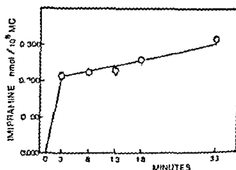


Fig 1 Fig 2

Fig 1 Incorporation of imipramine into isolated mast cells as a function of the concentration of imipramine in the incubation medium Incubations were carried out at 37 C for *30 min Ordinate Amount of imipramine incorporated expressed as nmol/10^6 mast cells* (MC) Abscissa Concentration of imipramine in the medium as molar concentration Means and S E of 3 expts incubated and determined in triplicate The value obtained for 5×10^{-7} M represents the mean value of 2 expts in triplicate

Fig 2 Incorporation of imipramine into isolated mast cells as a function of the incubation time Incubations were carried out at 37 C for 0 5 10 15 and 30 min with imipramine at 10^{-4} M Ordinate Amounts of imipramine incorporated expressed as *nmol/10^6* mast cells (MC) Abscissa Incubation time in min The values for the incubation time include the 3 min centrifugation preceding the washing of the cells Means and S E of 3 expts 2 in triplicate and 1 in duplicate

chlorpromazine HCl amitriptyline HCl and cocaine HCl (Medica Ltd) reserpine (Sigma) and guanethidine sulphate (Ismelin® Ciba AG) Reserpine was dissolved in *0 5 % citric acid* in 20 % ethanol in water all other drugs were dissolved in water Drug concentrations and amounts are given in terms of free bases Mast cells and leucocytes were counted in a Buerker chamber The morphology of the sediments was checked by electron microscopy (Philips EM 300 using conventional methods for fixation embedding sectioning and post-staining

Results

Incorporation of imipramine into pleural and peritoneal cells Imipramine was incorporated into mast cells and leucocytes *in vitro* At an exogenous concentration of 10 M 0.311 ± 0.013 nmol imipramine/10^6 mast cells was incorporated in 30 min at 37 C (mean ± S E of 23 expts in duplicate or triplicate) The amount of imipramine bound to leucocytes under corresponding conditions was *0.184 ± 0.009* nmol/10^6 leucocytes The difference in amounts of cell bound drug probably reflects a difference in size between mast cells and leucocytes and is indicative of a lack of specificity of the binding of imipramine at the concentration used

Incorporation of imipramine as a function of the exogenous concentration The amount of imipramine incorporated into mast cells in 30 min at 37° C increased in a sigmoidal fashion with increasing exogenous concentration between 5×10^{-7} M and 10^{-4} M Fig 1 The amount incorporated at 5×10^{-7} M was 0.059 ± 0.006 nmol/10^6 mast cells the corresponding figure at 10^{-4} M was 0.889 ± 0.056 nmol/10^6 mast cells. The ratio between cell bound drug (C_b) and the concentration of

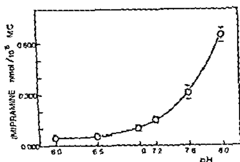


Fig 3

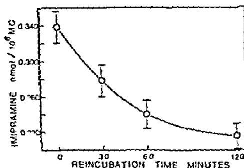


Fig 4

Fig 3 Effect of pH of the incubation medium on the incorporation of imipramine into isolated mast cells Incubations were carried out at 37 C for 30 min with imipramine at 10 M Phosphate buffer was used between 6 0—7 2 Tris-HCl buffer between 7 2—8 0 Ordinate Amounts of imipramine expressed as nmol/10^6 mast cells (MC) Abscissa pH of the medium Means and S E of 2 expts incubated and determined in triplicate The point at pH 7 2 was obtained after pooling the results obtained in the phosphate and Tris-HCl buffers

Fig 4 Effect of reincubation in drug free medium on the imipramine contents of mast cells previously incubated with imipramine at 10 M for 30 min at 37 C After 2 washes the cells were resuspended and incubated in a drug free medium for 0 to 120 min at 37 C Ordinate Amounts of imipramine remaining in the cells expressed as nmol/10^6 mast cells (MC) Abscissa Reincubation time in min Means and S E of 2 expts in triplicate

imipramine in the medium (C_o) decreased from roughly 120 to 10 as the concentration of imipramine was raised from 5×10^{-7} M to 10^{-4} M indicating a saturable process The C_b/C ratio did not decrease in a linear fashion with increasing exogenous concentration There was no appreciable change in the ratio between 5×10^{-6} M and 10^{-5} M indicating a sharp rise in the binding of imipramine to mast cells as the exogenous concentration was raised to this range

Incorporation of imipramine as a function of time The binding of imipramine as a function of time took place in two phases There was a very fast initial phase which was completed within a few minutes and which constituted 67 % of the maximal binding recorded This rapid phase was followed by a slow phase which proceeded linearly for 30 min and then tapered off At an exogenous concentration of 10 M 0 213 ± 0 009 nmol/10^6 *mast cells became bound during the 3 min centrifugation preceding the washing of the cells* (Fig 2) After this initial phase the incorporation progressed reaching a maximum of 0 314 ± 0 028 nmol/10^6 mast cells in 30 min whereafter there was a decline to 0 267 ± 0 033 nmol/10 mast cells in 60 min

Incorporation of imipramine as a function of pH As can be seen from Fig 3 the pH of the incubation medium markedly affected the binding of imipramine Barely detectable amounts of imipramine were incorporated at pH 6 0 at an exogenous concentration of 10 M Between pH 7 0 and 8 0 the amount of cellbound imipramine rose from 0 094 ± 0 006 at pH 7 0 to 0 648 ± 0 034 nmol/10^6 mast cells at

TABLE I Effect of some drugs on the incorporation of imipramine into isolated mast cells

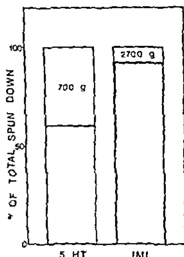
Drug added	Concentration M	Imipramine * of control
Guanethidine	$5\times10^{-}$	76 2 ± 11 4
Reserpine	$5\times10^{-}$	68 1 ± 10 9
Nortriptyline	10^{-5}	64 8 ± 4 7
Amitriptyline	10^{-5}	87 5 ± 11 3
Chlorpromazine	10^{-5}	64 1 ± 6 5
Cocaine	10^{-}	88 6 ± 11 8
2 Hydroxy imipramine	10^{-5}	85 5 ± 9 5
2 Hydroxy desipramine	10^{-5}	109 ± 10 2
Chlorimipramine	10^{-5}	60 5 ± 9 6
5 Hydroxytryptamine	10^{-5}	76 6 ± 7 3
5 Hydroxytryptamine	10^{-}	99 5 ± 8 2
5 Hydroxytryptophan	$5\times10^{-}$	98 9 ± 5 2

Incubations were carried out at 37 C with imipramine at 10^{-} M Drugs were added 30 min before the addition of imipramine whereafter incubations were continued for a further 30 min Incorporation in the presence of drug is expressed in % of that of control without drug Means and S E of 1–2 expts incubated and determined in triplicate

pH 8 0 This increase obviously reflects an increase in the nonionized species (i e more lipid soluble) over the ionized species as the pH of the medium is increased

Effects of temperature and some factors on the incorporation of imipramine The incorporation of imipramine into mast cells proved to be almost completely independent of the incubation temperature The incorporation achieved after incubation at 0 22 and 37° C was 0 257 ± 0 029 0 235 ± 0 012 and 0 246 ± 0 017 nmol/10^6 mast cells respectively (means and S E of 5 expts in triplicate) Calcium ions were not found to affect the binding of imipramine At Ca concentrations of 0 2 2 and 8 8 mM in the medium the amounts of imipramine incorporated were 0 368 ± 0 033 0 348 ± 0 041 and 0 363 ± 0 040 nmol/10^6 mast cells respectively (means and S E of 2 expts in triplicate) An addition of EGTA a chelator of calcium ions at 1 0 mM did not cause any change in the amounts incorporated Bovine serum albumine routinely added to the medium decreased the amount of imipramine bound to the cells Albumine at the standard concentration of 1 0 mg/ml caused a 21 % reduction in the amount of cell bound imipramine compared with the amount incorporated in the absence of albumine

Effect of drugs on the incorporation of imipramine The incorporation of imipramine was decreased by most of the drugs tested when present simultaneously with imipramine in the incubation medium (Table I) Guanethidine at 5×10^{-5} M reduced the amount of imipramine incorporated to 76 2 ± 11 2 % and reserpine at 5×10^{-6} M to 68 1 ± 10 9 % of the control level 5 HT at 10^{-4} M did not affect the incoporation of imipramine while at 10^{-5} M the amount of imipramine incorporated decreased to 76 2 ± 6 9 % of the control level Of the drugs structurally related to imipramine chlorimipramine chlorpromazine and nortriptyline all reduced the binding of imipramine while 2 hydroxy desipramine was without effect in this respect



ig 5 Distribution of imipramine and 5 hydroxytryptamine in subcellular fractions of isolated mast cells pre iously incubated with imipramine at 10^{-5} M Fractions ollected as described under Materials and methods The nts of imipramine and 5 HT are expressed as a % the total amount spun down *i e* the sum of the nounts found in the $350 \times g$ and $2700 \times g$ sediments he $350 \times g$ sediments is indicated by the shadowed area the $2700 \times g$ sediments by figures Means and S E of 4 expts. determined in triplicate

Effect of reincubation in drug free medium of cells preloaded with imipramine When mast cells previously incubated with imipramine at 10^{-5} M were washed and resuspended in a drug free medium 18 % of the cell bound imipramine was released during the first 30 min of reincubation The corresponding figures for 60 and 120 min fo reincubation were 29 and 36 % 5 HT seemed unable to displace imipramine from its binding sites since after 120 min of reincubation in the presence of 5 HT at 10 M only 4 % more imipramine was washed out compared with the control without 5 HT The release of cell bound imipramine was not exponential it tapered off after 1 h of reincubation and approached a level representing 60 % of the total drug incorporation which seemed to be fairly tightly associated with the cells (Fig 4)

Subcellular distribution of imipramine The subcellular distribution of imipramine and 5 HT in mast cells clearly differed Differential centrifugation carried out after freezing and thawing of a mast cell population previously incubated with imipramine produced two fractions a heavy sediment ($350 \times g$) consisting of partially broken cells cell debris and granules and a light sediment ($2700 \times g$) consisting mainly of membrane free granules The heavy sediment held 92 3 ± 0 64 % and the light sediment 7 75 ± 0 64 % of the total amount of imipramine spun down (Fig 5) The corresponding figures for the endogenous 5 HT content were 60 8 ± 3 7 and 39 2 ± 3 7 % Thus only a minor part of the cell bound imipramine was located in the membrane free mast cell granules The low affinity of imipramine for membrane free mast cell granules was further demonstrated by experiments with isolated granules In these experiments only barely detectable amounts of imipramine were incorporated into a batch of granules corresponding to 10×10^{6} isolated mast cells

Discussion

As judged from the present results, the binding of imipramine to isolated mast cells *in vitro* showed the following features

1 The binding of imipramine was unaffected by the incubation temperature the highest amounts of imipramine were actually incorporated at 0° C

2 The incorporation took place in two phases one of which was very rapid

3 The C_i/C_o ratio decreased in a nonlinear fashion as the concentration in the medium increased indicating a saturable multicompartmental process

4 The binding of imipramine increased with increasing pH of the medium, especially in the pH range 7 0—8 0

5 The binding of imipramine to mast cells was quite firm and the release of cell bound drug was not greatly altered by 5 HT About 40 % of the amounts bound to the cells was quite easily removed by washing

6 The subcellular distribution of cell bound imipramine was different from that of endogenuos 5 HT indicating a nongranular binding of the drug

7 Membrane free mast cell granules did not retain imipramine in amounts high enough to be detected by the present method

8 The binding of imipramine was reduced by several imipramine derivatives and tricyclic antideperssants as well as by 5 HT at high concentrations

Passive adsorption of imipramine seems to account for the binding of this drug to mast cells *in vitro* judging from the rapidness the lack of effect of temperature and the pH dependence of this process This is in agreement with earlier observations indicating a very rapid binding of imipramine to blood platelets (Boullin and O Brien 1968) rat brain subcellular particles (Weinstein *et al* 1971) and isolated rat lung (Junod 1972) Furthermore the binding of phenothiazines to erythrocytes is very rapid and is explained as being the result of passive adsorption (Seeman 1966 In analogy the binding of more than 0 200 nmol imipramine/10^6 mast cells in three minutes is not likely to be explained by anything other than simple adsorption to the cell membrane The lack of effect of temperature on the binding of imipramine to mast cells supports the idea of binding as being a passive process and is in agreement with the results obtained by Kwant and Seeman (1969) regarding temperature independence of binding of chlorpromazine to erythrocyte membranes The pH dependence of binding of imipramine to mast cells also parallel the results reported by Kwant and Seeman (1969) on chlorpromazine in erythrocyte membranes

The dissimilar subcellular distribution of imipramine and endogenous 5 HT in mast cells and the failure to demonstrate any significant binding of imipramine to isolated membrane free mast cell granules suggest that imipramine becomes attached to the cell membrane Incubation for 30 min with imipramine at 10[illegible] M resulted in the binding of 2 × 10[illegible] molecules of imipramine/1 mast cell Taking the mean diameter of these cells to be 15 μm the average membrane area occupied by one molecule of imipramine is roughly 400 Å If assuming that the cell membrane is smooth the concentration of imipramine within the membrane would be 0 0[illegible]

nol/l wet membrane These figures are in agreement with those regarding pheno hiazines in erythrocyte membranes (Seeman 1966 Kwant and Seeman 1969, See nan 1972) It thus seems that the assumption of imipramine acting at the level of he outer cellular membrane in central 5 HT neurons (Carlsson *et al* 1969) and lood platelets (see *e g* Paasonen 1973) is also valid for mast cells

The question as to whether imipramine in interfering with the operativeness of the membrane pump for monoamines in mast cells is bound to a specific carrier ite in the cell membrane cannot be definitely answered at the present time However as judged from present results imipramine seems to become bound to at least 2 different membrane sites This is indicated by the observations that 1) the binding of imipramine as a function of the exogenous concentration was multiphasic 2) the binding was composed of one rapid phase followed by a slower phase and finally 3) a portion of the cellbound drug was easily washed out while the remaining fraction seemed more tightly associated to the cells The possibility that one of the uggested multiple binding sites may represent a specific carrier site must not be disregarded The observation that the binding of imipramine was inhibited by 5-HT only at rather high concentrations does not rule out the possibility of a specific binding of imipramine to a carrier site as differences in the affinities of these two agents for a carrier site may be expected

Thus at high concentrations an unspecific binding of imipramine to the cell membrane would be expected This probably results in changes in the perme ability of the membrane as has been shown to occur with chlorpromazine (Frisk Holmberg 1971 for further ref see Seeman 1972) The enhanced efflux of 5 HT from mast cells after exposure to imipramine (Jansson and Pentula 1969) may thus be due to intracellular accumulation of sodium ions (Uvnas *et al* 1970) On the other hand the uptake of 5 HT into various *in vitro* models is affected by imi pramine at concentrations considerably lower than those routinely used in the present study (*i e* 10 M) Thus the uptake of 5-HT into mouse cerebral slices (Carlsson 1970) rat brain slices (Ross *et al* 1971) and blood platelets (Stacey 1961 Todrick and Tait 1969 Ahtee and Saarnivaara 1971) are all characterized by a 50 % inhibition of the 5 HT uptake being reached at an imipramine concentration around 5×10^{-7} M to 10^{-6} M This figure also seems to hold true for mast cells since preincubation with imipramine at 10^{-6} M effectively depresses the uptake of 5 HT (Jansson 1970) and a 50 % inhibition is reached with imipramine at around 5×10^{-7} M (Frisk Holmberg 1972) It is suggested that exposure of mast cells to imipramine at low concentrations *e g* 5×10^{-7} M at which considerable amounts of the drug still bind to the cells (present results) results in a specific binding of imipramine to sites in the cell membrane primarily affecting the operativeness of the amine carrier mechanism while at higher concentrations imipramine also binds to unspecific sites in the cell membrane further impairing the function of the cell membrane Experiments designed to test the suggestion of specific binding of imi pramine to a 5 HT carrier site in mast cells are under progress

References

AHTEE L J TUOMISTO E SOLATUNTURI and M K PAASONEN Effect of some phenothiazine derivatives and related agents on the accumulation and distribution of 5 hydroxytryptamine in blood platelets *Ann Med exp Fenn* 1968 *46* 429—434

AHTEE L and I SAARNIVAARA The effect of drugs upon the uptake of 5 hydroxytryptamine and metaraminol by human platelets *J Pharm Pharmac* 1971 *23* 495—501

BOLTLIN D J and R A O'BRIEN The binding of imipramine to the outer membrane of blood platelets *J Pharm Pharmac* 1968 *20* 583—584

CARLSSON A K FUXE and U UNGERSTEDT The effect of imipramine on central 5 hydroxytryptamine neurons *J Pharm Pharmac* 1968 *20* 150—151

CARLSSON A H CORRODI K FUXE and T HOKFELT Effect of antidepressant drugs on the depletion of intraneuronal brain 5 hydroxytryptamine stores caused by 4 methyl α-ethyl meta tyramine *Europ J Pharmac* 1969 *5* 357—366

CARLSSON A Structural specificity for inhibition of (14C) 5 hydroxytryptamine uptake by cerebral slices *J Pharm Pharmac* 1970 *22* 729—732

DA PRADA M and A PLETSCHER Isolated 5 hydroxytryptamine organelles of rabbit blood platelets physiological properties and drug induced changes *Br J Pharmac* 1968 *34* 591 –597

DINGELL J V F SULSER and J R GILLETTE Species difference in the metabolism of imipramine and desmethylimipramine (DMI) *J Pharmacol exp Ther* 1964 *143* 14—22

FRISK HOLMBERG M On the Mechanism of Chlorpromazine Induced Histamine Release from Mast Cells *Acta physiol scand* 1971 *83* 412—421

FRISK HOLMBERG M Inhibition of the Uptake of Biogenic Amines into Mast Cells by Tricyclic Psychoactive Drugs *Acta physiol scand* 1972 *85* 446—454

GRIPENBERG J S E JANSSON and T PARTANEN Incorporation of Reserpine into Isolated Rat Peritoneal Mast Cell Garnules in Vitro *Acta physiol scand* 1972 *86* 433—443

GRIPENBERG J Incorporation of Guanethidine into Isolated Rat Peritoneal Mast Cells and Mast Cell Granules in Vitro *Acta physiol scand* 1973 *88* 516—527

JANSSON S E and A PENTTILA Effect of pH temperature osmolarity and drugs on 5 hydroxytryptamine content and light and electron microscopic structure of rat mast cells in vitro *Exp Cell Res* 1969 *54* 367—376

JANSSON S E Uptake of 5 Hydroxytryptamine by Rat Peritoneal Mast Cells in Vitro *Acta physiol scand* 1970 *79* 484—492

JANSSON S E Uptake of 5 Hydroxytryptamine by Mast Cell Granules in Vitro *Acta physiol scand* 1971 *82* 35—46

JUNOD A F Accumulation of 14C imipramine in isolated perfused rat lungs *J Pharmacol exp Ther* 1972 *183* 182 –187

KWANT W O and P SEEMAN The membrane concentration of a local anesthetic chlorpromazine *Biochim biophys Acta* (Amst) 1969 *183* 530—543

LAGUNOFF D M T PHILLIPS O A ISERI and E P BENDITT Isolation and preliminary characterization of rat peritoneal mast cell granules *Lab Invest* 1964 *13* 1331—1344

PAASONEN M K Blood Platelets as a Model for Aminergic Neurons Pharmacology and the Future of Man Proc 5th Int. Congr Pharmacology San Francisco 1972 vol 4 pp 328–342 (Karger Basel 1973)

ROSS S B A L RENYI and S O OGREN A comparison of the inhibitory activities of iprindole and imipramine on the uptake of 5 hydroxytryptamine and noradrenaline in brain slices *Life Sci* 1971 10 (Part I 1267 –1277

SEEMAN P Membrane stabilization by drugs tranquilizers steroids and anesthetics *Int Rev Neurobiol* 1966 *9* 145—221

SEEMAN P The Membrane Actions of Anesthetics and Tranquilizers *Pharmacological Reviews* 1972 *24* 583 –655

SHASKAN E G and S H SNYDER Kinetics of serotonin accumulation into slices from rat brain relationship to catecholamine uptake *J Pharmacol exp Ther* 1970 *175* 404—418

SNYDER S H J AXELROD and M ZWEIG A sensitive and specific fluorescence assay for tissue serotonin *Biochem Pharmacol* 1965 *14* 831—835

STACEY R S Uptake of 5 hydroxytryptamine by platelets *Brit J Pharmacol* 1961 *16* 284—295

THON I L and P UVNAS Mode of Storage of Histamine in Mast Cells *Acta physiol scand* 1966 *67* 455 –470

UVNAS B and I L THON Isolation of "biologically intact mast cells *Exp Cell Res* 1959 *18* 512—520

UVNÄS B C H ÅBORG and A BERGENDORFF Storage of Histamine in Mast Cells Evidence for an Ionic Binding of Histamine to Protein Carboxyls in the Granule Heparin Protein Complex *Acta physiol scand* 1970 Suppl 336

TODRICK A and A C TAIT The inhibition of human platelet 5 hydroxytryptamine uptake by tricyclic antidepressant drugs The relation between structure and potency *J Pharm Pharmac* 1969 *21* 751—762

VANABLE J W A Ninhydrin Reaction Giving a Sensitive Quantitative Fluorescence Assay for 5 Hydroxytryptamine *Anal Biochem* 1963 *6* 393—403

WEINSTEIN H S VARON and E ROBERTS Effect of imipramine on the Na-dependent exchange retention of γ aminobutyric acid by mouse brain subcellular particles *Biochem Pharmacol* 1971 *20* 103—117

WEISSBACH H The estimation of serotonin in biological material *Stand Meth Clin Chem* 1961 *4* 197—203

YATES C M A TODRICK and A C TAIT Effect of imipramine and some analogues on the uptake of 5-hydroxytryptamine by human blood platelets in vitro *J Pharm Pharmac* 1964 *16* 460—463

Acta physiol scand 1974 92 66—83
From the Department of Physiology University of Umeå Sweden

Parallel Activation of Dynamic Fusimotor Neurones and a Climbing Fibre System from the Cat Brain Stem
II Effects from the Inferior Olivary Region

By

Torgny Jeneskog

Received 25 February 1974

Abstract

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Climbing fibre activity in the cerebellar paramedian lobule and hind limb dynamic fusimotor activation caused by electrical stimulation in the medial parts of the medulla were studied in halothane anesthetized cats Three regions influencing dynamic fusimotor neurones were found From one of these corresponding to the nucleus reticularis gigantocellularis descending activity proceeded in the contralateral ventrolateral funiculus From the other two corresponding to parts of the inferior olive and the pyramid respectively the spinal pathways were located in the contralateral dorsolateral funiculus The pathway from the olivary region seemed to be identical to a previously described dynamic facilitatory pathway activated in the rubral region Climbing fibre activity in a hind limb zone of the paramedian lobule was evoked with short latency from caudal parts of the principal olive but probably transsynaptically also from wider areas within the inferior olive On stimulation in the red nucleus orthodromic responses were found in one region overlapping the nucleus reticularis gigantocellularis and in another one situated in the caudal parts of the principal olive In this latter region postsynaptic activity was recorded also from the DLF SOCP Evidence for a convergence between this pathway and the one from the rubral region was obtained

In a previous paper (Jeneskog 1974) it was reported that electrical stimulation within a restricted mesencephalic region closely corresponding to the red nucleus simultaneously activated hind limb dynamic fusimotor neurones and climbing fibres destined for the D zone (Voogd 1969) of the cerebellar cortex Dynamic facilitation effects from the rubral region are known to proceed in the dorsolateral funiculus of the spinal cord contralateral to the central stimulus although neither the rubrospinal nor the corticospinal tracts are responsible (Appelberg and Jeneskog 1973, 1972) Appelberg and Molander (1967) reported however that dynamic spindle effects were elicited also from a medullary region homolateral to the rubral one and histologically localized to the inferior olivary complex On several grounds these authors suggested that the pathway from the rubral region passed through and relayed in the homolateral inferior olive on its way to the spinal cord Furthermore

Appelberg (1967) described a monosynaptic connection between these two brain stem sites, and showed that both areas on stimulation evoked climbing fibre activity in the cerebellar cortex

The medial medullary region of the lower brain stem was now further investigated with threshold mapping of areas giving dynamic spindle effects. Connections were analysed both orthodromically and antidromically between the mesencephalic and medullary regions and furthermore climbing fibre responses evoked from the medullary region were studied

The results support the suggestion of Appelberg and Molander (1967) that the rubral and inferior olivary regions are parts of the same descending system to dynamic fusimotor neurones and the hypothesis of Miller and Oscarsson (1970) suggesting that descending command signals for motor activities may be seen to the cerebellum via collateral to the inferior olivary neurones. This hypothesis has already been discussed in the previous paper (Jeneskog 1974)

A preliminary report of some of the results has appeared (Appelberg and Jeneskog 1973)

Methods

The results to be presented were obtained in experiments on 3 cats Nineteen of these were anesthetized with halothane and these same were also used in the investigation of rubral effects on dynamic fusimotor neurones and on climbing fibres to the paramedian lobule (Jeneskog 1974) The principal methods were described in that paper and only additional details will be given here

Stimulation in the medulla was performed via a stereotaxically guided grid of 3 or 4 platinum iridium wire electrodes of the same type as those described in the previous paper The grid was oriented medio-laterally in 10 expts and sagittally in 9 expts. Interelectrode distance was 1 mm and the grid was inserted through the brain stem at an angle of 15 to the vertical plane (tips forward) to avoid the bony tentorium When sagittal grids were used the electrodes were mounted so as to have their tips in the same horizontal plane when inserted into the brain stem. The electrodes were also used for recording of field and unitary potentials

The electrodes were made primarily as stimulating electrodes, and thus their characteristics as recording electrodes were not optimal However pre and post synaptic components and also cell spike activity were readily distinguished. The latency measurements concerning these components were largely made directly on the oscilloscope screen with a fast time base usually in the storage mode The sampling of several sweeps which were stored on to each other showed that the different components had stable latencies and made the measurements more reliable

Thirteen animals were anesthetized with pentobarbital (Nembutal 6% ACO) throughout the experiments, and were used for mapping antidromic field potentials evoked from the medulla and recorded in the rubral region. The operation in these experiments included two craniotomies one over the right mesencephalon and the other over the posterior part of the cerebellum to allow stereotaxic electrode penetrations to the right rubral and the right inferior olivary regions respectively In a thoracic (Th1 —Th13) laminectomy the dorsal lamina were removed over approximately one segment This permitted antidromic activation of the rubrospinal tract on surface stimulation of the left dorsolateral part of the spinal cord The technique made it possible to directly compare the extent of the antidromic field potential in the rubral region evoked from the homolateral medulla with the extent of the antidromic rubrospinal field evoked from the contralateral low thoracic spinal cord

For histological work the brain stems were treated as described previously (Jeneskog 1974) In experiments where lesions were performed to localize the spinal course of pathways the relevant part of the cord was removed with the vertebrae after the experiment After fixation in Holt and Hicks buffered formaldehyd solution the piece of cord was dissected out dehydrated embedded in paraffine and cut in serial sections 10 μm which were stained with Luxol fast blue

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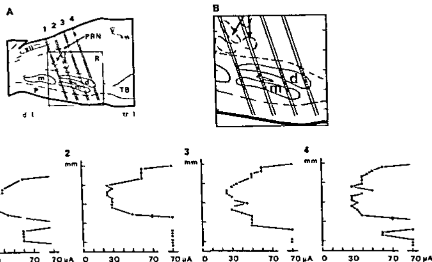


Fig 2 A is a camera lucida drawing of a parasagittal section approx 0 8 mm from the mid line through the lower brain stem from one experiment The 4 electrode tracks (1—4) are schematically indicated Tips were angled 15 in the rostral direction and mounted so as to be in the same horizontal plane The enclosed area is enlarged in B The bar in each track in B indicates the region from which climbing fibre responses were evoked in the contralateral D zone of the paramedian lobule in that experiment (≤ 70 μA single shock stim in tracks 1—2 ≤ 70 μA train stim. in tracks 3—4) Vertical mm scales correspond to the ordinates of diagrams C1—4 C The diagrams refer to similarly indicated tracks in A and show thresholds for evoking dynamic fusimotor activation plotted against depth in the medial medulla Vertical bars at the 70 μA line as in B Vertical segmented line bar in 2 indicates where a postsynaptic wave was recorded (the only track tested) on stimulation in the rubral region

the tracks were transferred to a standard series of transverse sections through the lower brain stem These diagrams (I—V) were drawn from histological sections through the medulla the intervals between them being approximately 1 mm In order to have the sections parallel to the electrode tracks the brain stem was cut at 15° to the vertical plane (*cf* Methods) The vertical lines (denoted a b and c in medio lateral direction) in the diagrams represent the electrode tracks and their thickened parts indicate the effective stimulating regions at each level The results of an individual experiment were projected on to the nearest diagrams in the illustrated series Dynamic fusimotor activation was detected indirectly by observing the response of primary muscle spindle afferents to the twitch test and sometimes also to linear extension of the muscle under control conditions as well as during repetitive stimulation in the medial medulla (*cf* Appelberg and Jeneskog 1968 1972)

The threshold for dynamic fusimotor activation from the medial medulla varied between experiments and was 15—45 μA in those experiments from which Fig 1 was constructed The bars in the diagrams show the extent of the stimulating regions as mapped with 70 μA stimulation Throughout the investigated parts of the medulla the effective low threshold region covered the inferior olivary com

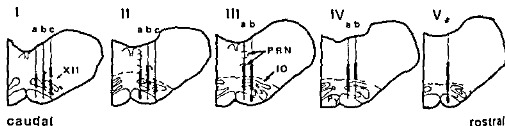


Fig 1 I—V are camera lucida drawings of transverse histological sections through the lower brain stem viewed from behind I is near the caudal border of the inferior olivary complex and the distance between successive diagrams is approx 1 mm The rostral pole of the inferior olivary complex is approx 0 5 mm rostrally to diagram V The parallel vertical lines (a b and c) in each diagram represent the electrode tracks and their thickened parts indicate low threshold regions (≤ 70 μA) for eliciting dynamic spindle effects Apart from the pyramid there are two regions one coinciding with the inferior olive and the other situated dorsally to this nuclear complex See text for further explanation

Abbreviations
CF climbing fibre CFR climbing fibre response CP cerebral peduncle d dorsal accessory olive D longitudinal cerebello-cortical zone DLF dorsolateral funiculus of spinal cord DLF SOCP dorsolateral spino olivocerebellar path HPT habenopeduncular tract IO inferior olivary complex L left m medial accessory olive MG medial geniculate body Mm mamillar body PAG periaqueductal gray substance PM paramedian lobule of posterior cerebellar lobe PRN paramedian reticular nucleus Pyr pyramid R right Ret reticular formation RN red nucleus SC superior colliculus S T I spindle twitch index TB trapezoid body II oculomotor nerve VI abducent nucleus XII hypoglossal nucleus and nerve

Results

Medullary stimulating regions for actuation of dynamic fusimotor neurones Spinal course of pathways

The medial parts of the medulla at the level of the inferior olive were explored for regions giving dynamic fusimotor activation when electrically stimulated During the experiments it was noted that also static fusimotor neurones could be influenced from certain rather dorsal points in the medial medulla but for the purpose of the present study only dynamic facilitatory areas were more fully analysed It was found that the mapping studies were greatly dependent upon the good condition of the preparation as well as of a careful setting of the anesthetic level within rather narrow limits This has earlier been described for corresponding studies concerning the rubral region (Appelberg and Molander 1967 Appelberg and Jeneskog 1972) Another difficulty was that the repetitive electrical stimulation used when delivered to regions dorsal to the inferior olivary complex often resulted in respiratory arrest even with quite low stimulating intensities It was noted however that when stimulatory effects in an experiment were stable when elicited from the rubral region they were also mostly stable from the medullary regions and vice versa

The combined results from detailed threshold mappings in 7 experiments are shown in Fig 1 In 6 of these experiments the medullary electrode grid was sagittally oriented but in order to facilitate comparisons with other figures in this paper

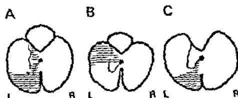


Fig 4 Camera lucida drawings of transverse histological sections through the spinal cord in the 13th thoracic segment from three different experiments Dotted areas indicate lesions performed during the operation and horizontal lines indicate lesions performed later during the experiments The lesion in A left both rubral and inferior olivary effects on dynamic spindle sensitivity uninfluenced The lesion in B simultaneously abolished such rubral and inferior olivary effects The lesion in C left the rubral dynamic facilitatory effects uninfluenced but abolished such effects from the dorsal medullary region

the regions from which climbing fibre responses were evoked with ⩽ 70 μA stimulation (train stimulation in tracks 3—4 single shock stimulation in tracks 1—2 see below) The vertical broken bar in diagram 2 indicates the region in which a postsynaptic wave could be recorded on stimulation in the rubral region The other tracks were not tested in that respect At rostral levels (Fig 2 C 3 and 4) two threshold minima are seen in the dorsal region one corresponding to the inferior olive and the other approximately 1 mm dorsally to this nuclear complex presumably within the nucleus reticularis gigantocellularis At caudal levels (Fig 2 C 1 and 2) there was only one threshold minimum in the dorsal region The extent of this minimum threshold area was however rather wide This might indicate that the two regions were so close to each other at this level that the threshold increase in between was undetectable The third low threshold region *i e* the one overlapping the pyramid was activated from two electrodes (1 and 4) in this experiment Effects from the pyramid were not thoroughly investigated but in one experiment it was verified that the effects descended in the contralateral dorsolateral funiculus of the spinal cord This is a confirmation of the findings of Vedel and Mouillac Baudevin (1970) who showed that electrical stimulation within the pyramid may elicit dynamic fusimotor activation

Activation of dynamic fusimotor neurones was usually studied simultaneously from the rubral region (Jeneskog 1974) and from the medial medullary region This made it possible to compare in the same experiment the effects obtained from each brain stem level The dynamic facilitatory effects from the inferior olivary and rubral regions are illustrated in Fig 3 A shows the response of a primary muscle spindle afferent to a muscle twitch under control conditions and B the response during repetitive stimulation (300 Hz) within the inferior olive The spindle twitch index (S T I *cf* Appelberg and Jeneskog 1972) increased from 96 in the control to 128 during stimulation indicating a strong elective dynamic fusimotor drive Fig 3 C and D show the same spindle afferent but now with stimulation in the rubral region to demonstrate the close similarity in effect from the two brain stem sites

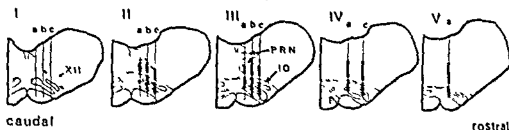


Fig. 5. Brain stem sections as described in Fig. 1. Thickened parts of the schematically drawn electrode tracks indicate orthodromically responding regions to right rubral stimulation. There are two regions almost throughout the investigated part of the lower brain stem, one coinciding with the inferior olive and the other situated dorsally to this nuclear complex. See text for further explanation.

It has earlier been demonstrated that dynamic facilitatory effects from the rubral region proceed in the dorsolateral funiculus (DLF) of the spinal cord contralateral to the stimulating electrode (Appelberg and Jeneskog 1969, 1972). This was confirmed in the present study by making spinal lesions in 5 expts. In 3 of these expts the lesion was made in the DLF in small steps between which the facilitatory effects on the spindle dynamic sensitivity were tested from both rubral and medullary regions. In 2 such expts (see example in Fig. 4 B), where the stimulating electrode tip was later shown to be in the inferior olive, the stimulatory effects disappeared simultaneously from the two brain stem sites. In the third case, with the medullary electrode tips in the dorsal region, the rubral effect disappeared first, and medullary effects were abolished only after further interruption of the lateral funiculus ventral to the DLF. In 2 other expts, lesions were made from the ventral side of the spinal cord, thus interrupting the ventral and ventrolateral parts first. In 1 of these expts, with the medullary stimulation taking place in the dorsal region, these effects were abolished by the ventrolateral lesion (Fig. 4 C), but the rubral effect was left uninfluenced. A later minute DLF lesion from the dorsal side abolished these rubral effects. In the remaining experiment, where the medullary stimulation was again performed in the inferior olive, a ventral and ventrolateral spinal lesion left both rubral and medullary effects uninfluenced (cf. Fig. 4 A).

In summary, at inferior olivary levels of the medial lower brain stem three sites were found which on electrical stimulation elicited dynamic spindle effects. From the ventral site, i.e. the pyramid, effects proceeded in the contralateral dorsolateral funiculus. From the region comprising the inferior olivary complex, effects also proceeded in the contralateral dorsolateral funiculus of the spinal cord, and the responsible pathway could not be separated by minute spinal lesions from the DLF pathway mediating effects from the rubral region. On the other hand, effects from the medial medulla dorsally to the inferior olive were shown to proceed in the ventrolateral parts of the contralateral spinal cord, and this pathway could thus easily be separated from the DLF pathway activated in the rubral region.

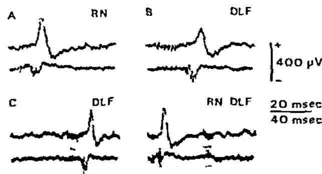


Fig 6 Upper trace in all records shows the climbing fibre response recorded with a surface electrode in a single point in the D zone of the left paramedian lobule Lower trace in all records is from one point in the right inferior olive (track IIIa of Fig 5 in ventral area) A shows the responses recorded on stimulation in the right rubral region (3 shocks at 600 Hz) and B the response to stimulation of the left DLF in Th13 (6 shocks at 600 Hz) Time scale 20 ms for A and B Left part of C shows same as B but with a slow time base In the right part of C is demonstrated that both the CFR and the inferior olivary response are totally depressed, when the DLF-stimulation is preceeded (approx. 50 ms) by a conditioning stimulus to the rubral region Time scale 40 ms to both parts of C Amplitude calibration applies to all records.

Orthodromic responses in the medial medulla evoked from the homolateral rubral region

Appelberg (1967) found that single shock stimulation in the rubral region which elicited dynamic fusimotor activation also evoked responses in the homolateral inferior olivary nucleus This matter was further studied in the present series and two orthodromically responding regions were disclosed in the medial medulla both homolaterally to the rubral stimulation Responses in both regions could be evoked on single shock stimulation in the rubral region but they were mostly studied with train stimulation (3 shocks at 600 Hz) because climbing fibre responses (CFRs) in the paramedian lobule (PM) were studied simultaneously (Jeneskog 1974)

Fig 5 is a summarized series of diagrams through the lower brain stem illustrating the two orthodromically responding regions found in 11 expts as thickened parts of the schematically drawn electrode tracks It should be noted that no orthodromic responses in either region could be recorded at level I i e where only dorsal and medial accessory parts of the inferior olivary complex are left The dorsal region corresponds anatomically to the nucleus reticularis gigantocellularis and the ventral region to the inferior olivary complex The responses in the two regions showed different time characteristics but had otherwise the same general appearance They usually consisted of an early positive negative spikelike deflection and a later negative wave At rostral levels in both regions the early component was prominent and the later wave was comparatively small In some recording points particularly in the caudal parts of both regions only the late negative wave was recorded The time to peak positivity of the early component was about 1 5 ms and to the beginning of the later negative wave about 2 5 ms for respones recorded in the dorsal region The corresponding values for responses in the inferior olive were 1 9—2.3 ms to peak positivity of the first component and 3 6—4 5 ms to the beginning of the later negative wave which itself lasted for several milliseconds The components

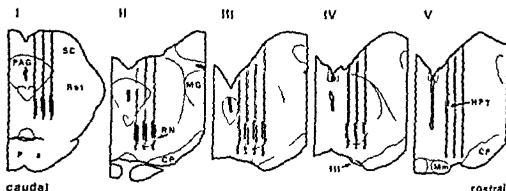


Fig 7 I—V are camera lucida drawings of transverse histological sections through the brain stem viewed from behind I is at the caudal border of the red nucleus and the distance to successive diagrams is approx 1 mm The rostral pole of the red nucleus is between diagrams IV and V The parallel vertical electrode tracks are marked in each diagram Black bars on the left side of each track indicate an antidromic field potential evoked from the right dorsal medullary region Black bars on the right side of each track indicate an antidromic field potential evoked from the right inferior olivary region No antidromic fields could be recorded from either medullary region at level V i e rostrally to the red nucleus Level IV was investigated with only dorsal medullary stimulation

of the responses were interpreted as was done for spinal motor nuclei by Brooks and Eccles (1947) These authors considered the early initially positive spike like component as the incoming presynaptic volley and the later negative wave as signs of postsynaptic activity The time from the peak of the early positivity to start of the slow negative wave was attributed to a true synaptic delay In the present experiments on the inferior olivary region this time difference was 1 7—2 5 ms which is quite long but agrees with previous findings (Grant and Oscarsson 1966 referring to a personal communication from M Ito Armstrong and Harvey 1966 Appelberg 1967) that synaptic transmission through the inferior olive is slow Riding on the negative wave there were in both medullary regions often one or several negative spikes presumably representing synaptically evoked cell spikes The postsynaptic nature of the late negative wave is further indicated in Fig 6 The upper trace in each part is the CFR recorded with a surface electrode in the D zone (Voogd 1969) of the left PM (cf Jeneskog 1974) while the lower trace shows the response evoked in the right inferior olive (track IIIa of Fig 5 ventral area) Fig 6 A shows the responses to right rubral stimulation and Fig 6 B the responses to activation of the DLF SOCP (Larson Miller and Oscarsson 1969 Jeneskog 1974) in the low thoracic (Th13) region Fig 6 C shows that the negative wave in the inferior olive (the presumed postsynaptic wave) arising from DLF stimulation was virtually absent along with the CFR (right part of Fig 6 C) when this stimulation was conditioned by a preceding (approx 50 ms) stimulation to the rubral region The postsynaptic wave caused by the rubral stimulus however remained unchanged From this inferior olivary point a CFR could be evoked in the D zone of the left PM with less than 15 μA single shock stimulation (see below)

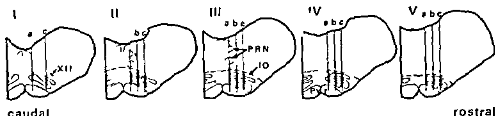


Fig 8 Brain stem sections as described in Fig 1 Thickened parts of the schematically drawn electrode tracks indicate low threshold region ($\leqslant 70\ \mu A$) for evoking climbing fibre responses in the D zone of the contralateral paramedian lobule At levels II and III single shock stimulation at levels I IV and V train stimulation (3 shocks at 600 Hz)

The latency to the onset of the CFR evoked from the rubral region was 7 8—8 6 ms (Jeneskog 1974) and to the onset of the late wave in the inferior olive 3 6—4 5 ms giving a time difference of about 4 ms This time difference corresponds well to the latency of surface recorded CFRs with stimulation within the inferior olive (3 7—4 3 ms), as measured in the present experiments The time difference between the CFR and the negative inferior olivary wave as evoked from the DLF of the spinal cord was also about 4 ms and this fact along with the disappearance of the inferior olivary wave in the conditioning test experiments indicates that it represents postsynaptic activity in the same elements (CF neurones) as those activated from the rubral region (*cf* Miller Nezlina and Oscarsson 1969 who showed that single Purkyne cells in the cerebello-cortical D zone were activated from the rubral region and from DLF SOCP) Similar latency differences between inferior olivary focal potentials and surface recorded CFRs were noted by Armstrong and Harvey (1968) on stimulation of hind limb nerves

Coincidence between extent of antidromic field potentials in the rubral region to dorsal medullary and inferior olivary stimulation

Connections between the rubral region and the homolateral lower brain stem were studied in a separate series of 13 expts mainly by mapping antidromic field potentials in the rubral region evoked by medullary stimulation The antidromic nature of the responses was indicated by their ability to follow high frequencies of stimulation Furthermore negative spikes behaving in an all or none manner were occasionally recorded at rostral levels of the red nucleus These spikes had constant and short latencies and followed short trains of stimulation at frequencies as high as 600 Hz and were interpreted as antidromically evoked cell spikes In all these experiments the rubrospinal tract was also activated antidromically in the low thoracic (Th13) cord and thus antidromic fields evoked from the medulla could be directly correlated to the red nucleus (*cf* Methods and Jeneskog 1974)

Fig 7 summarizes all the results on 5 equally spaced transverse histological sections through the brain stem at the level of the red nucleus Antidromic fields

recorded on stimulation within the dorsal medulla (12 penetrations in 5 expts) are shown on the left side of each track and the antidromic fields recorded on inferior olivary stimulation (9 penetrations in 4 expts) on the right side of each track The antidromic fields are seen to coincide closely at all rubral levels It should be especially noted that no antidromic fields were recorded at the mesencephalic level corresponding to diagram V i e rostrally to the red nucleus This level was penetrated in 1 expt with dorsal medullary stimulation and in 1 expt with inferior olivary stimulation and antidromic field potentials were readily recorded at more caudal levels in these experiments The level of diagram IV was not investigated in the experiments with inferior olivary stimulation, and thus it is not known whether or not the rostral limit of the antidromic field evoked from the olive is identical to the one from the dorsal medullary region

In the remaining 4 expts of this series the mesencephalic electrodes were kept stationary in the responding rubral region and the medullary regions giving an antidromic field potential were mapped out Two medullary areas were disclosed and the similarity between the extent of these areas and the orthodromically responding regions described in the previous section was very close as also in the respect that no antidromic field potentials were recorded in the rubral region from stimulating sites as caudal as level I of Fig 5

The latency of the antidromic field potentials was about 1 5 ms when evoked from the dorsal medulla and about 2 0 ms when evoked from the inferior olivary region These values agree with those of the presynaptic spike latency to rubral stimulation as described previously

The antidromically responding rubral region corresponds very closely to the rubral region giving dynamic fusimotor activation as described earlier (Appelberg and Molander 1967 Appelberg and Jeneskog 1972 Jeneskog 1974) and to the rubral region evoking CFRs in the D zone of the cerebellar cortex (Jeneskog 1974)

Considered together the results obtained in the studies of ortho- and antidromic connections between the rubral region and the medulla indicate that there exist monosynaptic connections from the rubral region to 2 different parts of the homolateral lower brain stem one to the inferior olive and one to an area dorsal to this structure possibly the nucleus reticularis gigantocellularis

Climbing fibre activation from the inferior olivary region Convergence from descending and ascending paths

Climbing fibre responses in the left paramedian lobule of the posterior cerebellar lobe caused by electrical stimulation in the right medullary region were studied in a total of 16 expts In the surface recordings employed the same criteria as described by Jeneskog 1974 were used to identify an evoked potential as a CFR

It was described by Jeneskog (1974) that CFRs were evoked in the D zone of the cerebellar cortex on stimulation in the same rubral region which also activated hind limb dynamic fusimotor neurones and for that reason mainly D zone CFRs evoked from the medulla were studied

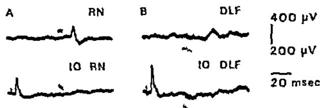


Fig 9 A upper trace shows the climbing fibre response evoked in the D zone of the left paramedian lobule on stimulation in the right rubral region (3 shocks at 600 Hz) Lower trace shows interaction on single shock stimulation in the right inferior olive (interval approx 50 ms) B upper trace shows the climbing fibre response (same recording point as in A) evoked on stimulation of left DLF (6 shocks at 600 Hz) Lower trace shows interaction from right inferior olive (interval approx 40 ms) Amplitude calibration is 400 μV for A and 200 μV for B Time scale applies to all records

Such responses could be evoked from certain areas in the investigated areas of the medial medulla covering in the rostro caudal direction approximately the whole inferior olivary complex Fig 8 summarizes the results from 23 electrode grid penetrations in the 16 expts The thickened parts of the schematically drawn electrode tracks indicate at each level the effective stimulating region which is seen to coincide complete with the inferior olivary complex The results of an individual experiment were projected on to the nearest diagrams in the series of transverse sections through the lower brain stem It should be noted that no CFRs were evoked from the region of the nucleus reticularis gigantocellularis *i e* the dorsal region responding orthodromically to rubral stimulation as described in a previous section The threshold for evoking a CFR varied between experiments and with the rostro caudal level of the electrode and was at the level of diagrams II and III as low as 10 μA with single shock stimulation Caudally (diagram I) as well as rostrally (diagrams IV and V) the threshold was higher and train stimulation was needed ($\geqslant 40$ μA 3 shocks at 600 Hz) to evoke a CFR The bars in the diagrams of Fig 8 show the extent of the effective region as mapped with 70 μA stimulation (single shock stim at levels II and III train stim at levels I IV and V)

The shortest latency of the evoked potential from the inferior olivary region was as measured in 5 expts 3 7—4 3 ms (mean 4 0 ms) These values were obtained on stimulation at levels II and III *i e* where the threshold for evoking a CFR was lowest From the other levels the latency was 1 5—2 ms longer but it could sometimes on raising the stimulating intensity be decreased abruptly by this value to about 4 ms

Convergence from the descending path activated in the rubral region (Jeneskog 1974) and the ascending DLF SOCP activated in the 13th thoracic segment of the spinal cord on to those inferior olivary CF neurones which evoked a D zone CFR on electrical stimulation within the nucleus was indicated by the results of the conditioning test procedure described in the previous paper (Jeneskog 1974 see also Miller *et al* 1969) This procedure takes into account the heavily depressed excitability of inferior olivary neurones up to about 100 ms after they have dis

charged (Armstrong and Harvey 1966, *cf* also Jeneskog 1974) Fig 9 is from such an experiment In the upper trace of Fig 9 A is shown the D zone CFR in the PM evoked from the rubral region The lower trace demonstrates that a preceeding (approx 50 ms) stimulation of the inferior olive (track IIIa in Fig 8 threshold for evoking a CFR from that point on single shock stimulation was < 20 μA) totally abolished the response to rubral stimulation The upper trace in Fig 9 B shows the CFR evoked from the DLF (Th13) and its total depression to a preceeding (approx 40 ms) stimulus of the same inferior olivary point as before

Climbing fibre responses in the contralateral D zone of the cerebellar paramedian lobule were thus evoked from wide areas within the inferior olive The shortest latency and the lowest threshold were however, found when the stimulating electrode was at the level of the caudal parts of the principal olive

Discussion

A previous paper (Jeneskog 1974) dealt with the activation of hind limb dynamic fusimotor neurones and of climbing fibres to the D zone (Voogd 1969) of the cerebellar cortex following stimulation in the rubral region It was demonstrated that the effective low threshold regions for eliciting each type of effect were remarkably similar in their extent and chiefly confined within the borders of the red nucleus It was suggested that both effects were the result of activation of a single descending system Such an organization would support the hypothesis of Miller and Oscarsson (1970) suggesting that descending command signals for motor activities may be sent via collaterals to inferior olivary neurones which inform the cerebellar cortex via climbing fibres An interaction was demonstrated between climbing fibre responses evoked from the rubral region and from the DLF SOCP the hind limb component of which projects to the cerebello cortical D zone (Larson *et al* 1969) This led to the conclusion that the descending pathway to the cerebellum relayed on the same inferior olivary neurones as were also activated by the DLF SOCP Results from an investigation of the inferior olivary region with regard to dynamic facilitatory and climbing fibre activating effects could give further support to or possibly disprove the above mentioned interpretations of earlier results

The present observations on the medial medullary region were made partly in the same experiments as those reported previously (Jeneskog 1974) Many of these experiments were designed in such a way that the effect of central stimulation on muscle spindle sensitivity from the two brain stem regions climbing fibre responses evoked from these same areas and the connections between mesencephalic and medullary regions could be studied in the same experiment By this procedure the suggestion of Appelberg and Molander (1967) that the rubral and inferior olivary regions are parts of the same descending system to dynamic fusimotor neurones could be tested

The results have been presented in three parts: 1) dynamic fusimotor activation from the medial medulla, 2) connections between these parts of the brain stem and the rubral region and 3) climbing fibre activation from the inferior olivary region, and they will be discussed in that order.

Dynamic fusimotor neurones were found to be activated from quite wide areas in the investigated parts of the medial medulla, as judged by the responses of primary muscle spindle afferents to central stimulation during extension and twitch tests of the muscle studied. However, with the threshold mappings employed, 3 low threshold regions were disclosed. One of them was situated within the pyramid, another covered the inferior olivary complex, and the third region was situated dorsally to this structure. Effects evoked on stimulation in the pyramid were shown to proceed in the contralateral dorsolateral funiculus of the spinal cord and were thus presumbaly mediated by corticospinal tract fibres. These observations confirm the results of Vedel and Mouillac-Baudevin (1970). These authors studied fusimotor effects from the sensorimotor cortex and also demonstrated that stimulation in the pyramid at the inferior olivary level may elicit dynamic fusimotor activation.

The other two low threshold regions, situated within and dorsal to the inferior olive respectively, were often confluent and only one threshold minimum was disclosed. In some cases, however, a small but definite threshold increase occurred at the dorsal border of the inferior olivary complex. This fact, as well as the results from some other tracks where only the one or the other region was activated, indicated that two descending systems directed to dynamic fusimotor neurones were activated simultaneously. This possibility was verified in experiments using spinal lesions to locate pathways in the cord. It was demonstrated that dynamic facilitatory effects from the region dorsal to the inferior olivary complex proceeded in the ventrolateral parts of the spinal cord, contralateral to the stimulating electrode. This dorsal medullary region seemed to coincide anatomically with the nucleus reticularis gigantocellularis of the medulla (see Brodal 1957, Fig. 1), whose descending fibres are known to run bilaterally in the ventral parts of the lateral funiculi (Nyberg-Hansen 1966). This author described furthermore degenerating fibres in the homolateral ventral funiculus following electrolytical lesions within the nucleus reticularis gigantocellularis, but concluded that these fibres were only passing and originated in the pontine reticular formation. Vedel and Mouillac-Baudevin (1969) reported that during light halothane anesthesia stimulation in the pontine reticular formation might elicit either static or dynamic fusimotor activation. Pontine reticular fibres are known, however, to descend strictly homolaterally (Brodal 1957, Nyberg-Hansen 1966) and seem to be ruled out as mediators of the presently observed dynamic effects because the stimulating electrode in these experiments was contralateral to the intact parts of the spinal cord. It is therefore suggested that the dynamic facilitatory effects from the dorsal medullary region were mediated by reticulospinal fibres originating the nucleus reticularis gigantocellularis.

Vedel and Mouillac-Baudevin (1969) described only static fusimotor effects from

this region but Appelberg and Jeneskog (1972), on studying mesencephalic fusimotor control noted that stimulation in a ventral mesencephalic region could evoke static or mixed static/dynamic but sometimes also purely dynamic, effects on spindle afferents. These effects were abolished by a ventral and ventrolateral spinal lesion and it was suggested that the spinal pathway consisted of reticulospinal fibres originating in the *nucleus reticularis gigantocellularis*. Static fusimotor effects were also elicited from the dorsal part of the dorsal medullary region on occasions in the present series as briefly mentioned in the Results section.

The dynamic effects elicited from stimulation in the nucleus reticularis gigantocellularis as well as the orthodromic and antidromic connections demonstrated between this region and the rubral region would indicate that stimulation *in the* rubral region might elicit dynamic fusimotor activation also via a ventrolateral spinal pathway. This has however not been demonstrated. On the contrary, when only the DLF *contra*lateral *to* the stimulating site in the rubral region is interrupted all dynamic facilitatory effects from this central site are abolished (*see* also Appelberg and Jeneskog 1969). The reason for these seemingly paradoxical result *remains* to be elucidated.

The dynamic spindle effects evoked from the medullary region coinciding anatomically *with* the *inferior olivary complex* were shown to proceed in the dorsolateral funiculus of the spinal cord contralateral to the stimulating electrode. The spinal pathway mediating these effects could not be separated by *minute* spinal lesions from the pathways giving dynamic facilitatory effects from the rubral *region* (Appelberg and Jeneskog 1969, 1972). In agreement with Appelberg and Molander (1967) as well as with Appelberg and Jeneskog (1972) it is therefore suggested that the rubral and inferior olivary regions are parts of the same descending system. The origin of these dorsolateral spinal fibres to dynamic fusimotor *neurones* however still remains unknown. Rubrospinal or corticospinal fibres are not *responsible* (Appelberg and Jeneskog 1969, 1972). The inferior olivary neurones seem *to project* *only to the cerebellum* (Brodal 1940) and it has been demonstrated that *total* cerebellectomy does not abolish the dynamic spindle effects *from the* rubral *region* (Appelberg and Molander 1967). Probably the pathway activated *in the* rubral region merely passes right through the homolateral inferior olivary *region* (although collaterals seem to branch off, see below) and then relays in the lower *brain stem* on neurones which are the origin of the spinal pathway.

A monosynaptic connection between rubral and *inferior* olivary regions giving dynamic fusimotor activation was described by Appelberg (1967) and such connections were further studied in the present investigation. Postsynaptic activity *could* be recorded in two homolateral medullary regions on stimulation *within the* rubral region. One of these medullary regions coincided anatomically with the *inferior olive* and the other was situated just dorsally to this *nuclear complex*. The possibility that it should in fact be a single region *seems to be contradicted* by the different time characteristics of the orthodromic responses as well as *by the* fact that stimulation in the dorsal medullary region did not evoke climbing *fibre responses* in the cere-

bello-cortical D zone This should have been the case if the rubral fibres divided to give one branch to the dorsal region and another thin branch to the inferior olive The dorsally responding region coincided fairly well with the nucleus reticularis gigantocellularis However a descending connection to this nucleus from the rubral region has not been described previously although the present experiments with antidromic field potential mapping indicate that the cells of origin of this descending path also lie within the borders of the red nucleus Edwards (1972) described a connection from the mesencephalic reticular formation to the nucleus reticularis gigantocellularis using a protein transport tracing method but did not specify the actual origin of fibres in the mesencephalon

The ventral responding region totally overlapped the rostral and middle parts of the inferior olive However in the most caudal parts where only the dorsal and medial accessory parts of the nuclear complex are left no orthodromic responses were recorded The largest postsynaptic potentials were found in the region from the caudal parts of the principal olive and extending about 1 mm rostrally Further rostrally within the olive the postsynaptic wave was small but the presynaptic component was more prominent These observations indicate that the relay cells activated from the rubral region are located at the level of the caudal parts of the principal olive Antidromic field potentials from this inferior olivary region could be recorded within but not rostrally to the red nucleus and it is therefore suggested that a rubro-olivary tract was studied

Climbing fibre responses in the left cerebello-cortical D zone could be evoked from rather wide areas of the inferior olivary complex From rostral inferior olivary levels and also from the part caudal to the principal olive the latency of the climbing fibre response was quite long and train stimulation was necessary to evoke it at threshold strength On raising the stimulating intensity to well above the threshold however the latency decreased abruptly by about 1 5 ms Only in the region of the caudal parts of the principal olive was the latency short also on threshold stimulation and then comparable to that of the direct activation of the cells giving rise to the climbing fibre response (Eccles Llinas and Sasaki 1966) or with the antidromic latency in the inferior olive on surface stimulation of the cerebellum (Armstrong and Harvey 1966) In this same region furthermore the lowest threshold for evoking a D zone climbing fibre response was found The observed higher threshold and longer latency for the CFR when evoked from the rostral and very caudal inferior olivary parts indicate that the CF neurones were transsynaptically activated from these areas Considering the convergence demonstrated from the DLF SOCP and the red nucleus for the D zone climbing fibre responses (Jeneskog 1974) it is significant that a suitably timed presumably postsynaptic wave could be recorded in this specific inferior olivary region not only on rubral stimulation but also on activation of the hind limb component of the DLF SOCP This wave showed the same interaction characteristics as did the surface recorded climbing fibre responses Although D zone responses from the inferior olivary region were mainly studied it was found that other areas of the paramedian lobule also showed

climbing fibre activation following inferior olivary stimulation especially when the electrode was rather rostral within the nuclear complex. However from the region where 1) the latency of the climbing fibre response was shortest 2) the threshold for evoking a climbing fibre response was lowest and 3) a pronounced postsynaptic wave could be picked up on rubral stimulation, the climbing fibre response was limited to the D zone provided that the stimulating intensity was not raised too much above the threshold From these facts it is suggested that the rubro-olivary tract monosynaptically and the DLF SOCP polysynaptically (Larson *et al* 1969) activate inferior olivary neurones located at the level of the caudal parts of the principal olive which neurones project to the D zone of the contralateral cerebellar cortex as climbing fibres

The low threshold medullary region for eliciting hind limb dynamic fusimotor effects via a dorsolateral spinal pathway except for the one presumably activating corticospinal fibres extended throughout the inferior olivary region Even in the most caudal parts behind the principal olive the threshold remained low A postsynaptic wave could be recorded on rubral stimulation at the level of the caudal parts of the principal olive but not further caudally Furthermore direct activation of cells (or fibres) ending as climbing fibres in the D zone of the cerebellar cortex could be performed only at caudal principal olivary levels This might indicate that the descending pathway to dynamic fusimotor neurones passes from the rubral region right through the homolateral inferior olivary region while giving off collaterals to the principal olive The pathway would then cross over to the other side of the brain stem very caudally because it is known (Appelberg and Jeneskog 1969) that already at high cervical levels (C1) it has achieved a dorsolateral spinal course contralateral to the central stimulating site

The results discussed seem to fully support the suggestion of Appelberg and Molander (1967) that stimulation in the rubral and the inferior olivary regions activate at different levels the same descending system to hind limb dynamic fusimotor neurones The results are also consistent with the hypothesis of Miller and Oscarsson (1970) suggesting that the cerebellum via inferior olivary climbing fibres might be informed of descending motor command signals

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References

APPELBERG B A rubro-olivary pathway II Simultaneous action on dynamic fusimotor neurones and the activity of the posterior lobe of the cerebellar cortex. *Exp Brain Res* 1967 3 382—390

APPELBERG B and T JENESKOG Extension and twitch of muscle as tests of muscle spindle static and dynamic sensitivities *Life Sci* 1968 7 Part I 1277—1282

APPELBERG B and T JENESKOG A dorso-lateral spinal pathway mediating information from the mesencephalon to dynamic fusimotor neurones. *Acta physiol scand* 1969 77 159—171

APPELBERG B and T JENESKOG Mesencephalic fusimotor control. *Exp Brain Res* 1972 15 97—112.

APPELBERG B and T JENESKOG Parallel activation from the cat brain stem of hind limb dynamic fusimotor neurones and climbing fibres to the cerebellar paramedian lobule *Brain Res* 1973 *59* 229—233

APPELBERG B and C MOLANDER A rubro-olivary pathway I Identification of a descending system for control of the dynamic sensitivity of muscle spindles *Exp Brain Res* 1967 *3* 372—381

ARMSTRONG D M and R. J HARVEY Responses in the inferior olive to stimulation of the cerebellar and cerebral cortices in the cat. *J Physiol* (Lond) 1966 *187* 553—574

ARMSTRONG D M and R. J HARVEY Responses of a spino-olivo-cerebellar pathway in the cat. *J Physiol* (Lond) 1968 *194* 147—168

BRODAL, A. Experimentelle Untersuchungen uber die Olivo-Cerebellare Lokalisation. *Z ges Neurol Psychiat* 1940 *169* 1—153

BRODAL, A *The Reticular Formation of the Brain Stem* Anatomical aspects and functional correlations The Henderson trust lectures Oliver and Boyd Edinburgh and London 1957

BROOKS C McC and J C ECCLES Electrical investigation of the monosynaptic pathway through the spinal cord *J Neurophysiol* 1947 *10* 251—274

ECCLES J C R. LLINAS and K SASAKI The excitatory synaptic action of climbing fibres on the Purkinje cells of the cerebellum *J Physiol* (Lond) 1966 *182* 268—296

EDWARDS S B Descending projections of the midbrain reticular formation of the cat an experimental study using a "protein transport tracing method *Anat Rec* 1972 *172* 305

GRANT G and O OSCARSSON Mass discharges evoked in the olivo-cerebellar tract on stimulation of muscle and skin nerves *Exp Brain Res* 1966 *1* 329—337

JENESKOG T Parallel activation of dynamic fusimotor neurones and a climbing fibre system from the cat brain stem. I Effects from the rubral region *Acta physiol scand* 1974 *91* 223—242

LARSON B S MILLER and O OSCARSSON Termination and functional organization of the dorsolateral spino-olivocerebellar path *J Physiol* (Lond) 1969 *203* 611—640

MILLER S N NEZLINA and O OSCARSSON Climbing fibre projection to cerebellar anterior lobe activated from structures in midbrain and from spinal cord *Brain Res* 1969 *14* 234—236

MILLER, S and O OSCARSSON Termination and functional organization of spino-olivocerebellar paths In *The Cerebellum in Health and Disease* Eds W S Fields and W D Willis Jr W H Green St. Louis Mo 1970 172—200

NYBERG-HANSEN R. Functional organization of descending supraspinal fibre systems to the spinal cord Anatomical observations and physiological correlations *Ergebn Anat Entwickl Gesch* 1966 *39* Heft 2 1—48

VEDEL J P and J MOUILLAC BAUDEVIN Etude fonctionnelle du controle de l activite des fibres fusimotrices dynamiques et statiques par les formations reticulees mesencephalique pontique et bulbaire chez le chat. *Exp Brain Res* 1969 *9* 325—345

VEDEL J P and J MOUILLAC BAUDEVIN Controle pyramidal de l activite des fibres fusimotrices dynamiques et statiques chez le chat *Exp Brain Res* 1970 *10* 39—63

VOOGD J The importance of fibre connections in the comparative anatomy of the mammalian cerebellum In *Neurobiology of Cerebellar Evolution and Development* Ed. R. Llinas AMA, Chicago 1969 493—514

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From the Institute of Physiology University of Oslo and The Department of Radiology Rikshospitalet Oslo Norway

Changes in Pulmonary Vascular Volumes after Induced Intravascular Aggregation of Blood Platelets

By

J VAAGE P AARSETH I ENGE and J HOGNESTAD

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Abstract

VAAGE J, P AARSETH I ENGE and J HOGNESTAD *Changes in pulmonary vascular volumes after induced intravascular aggregation of blood platelets* Acta physiol scand 1974 92 84—94

The volumes of intra and extravascular fluid compartments in lungs have been studied in cats after intravascular aggregation of blood platelets induced by collagen infusions Such infusions give marked pulmonary pressor responses A biopsy technique with removal of isolated lobes after isotopelabelling of the blood indicated an increased vascular volume during the response to a collagen infusion while the extravascular water content remained unchanged Angiographic observations revealed that the increased vascular volume is due to distension of large arteries while the veins have a somewhat reduced dimension These findings are interpreted as being caused by a precapillary vasoconstriction no marked changes on capillary pressure and with passive arterial dilatation upstreams to the constricted segments Venous narrowing might be active or due to some passive recoil

Microembolization of the lungs with blood platelet aggregates induces constriction of the smooth muscle cells in pulmonary resistance vessels as well as in the airways These effects are probably initiated by vasoactive substances released from the blood platelets during their aggregation (Bo and Hognestad 1972, Vaage Bo and Hognestad 1973 1974 Radegran and McAslan 1972) There is scarce information available however about the effect of such microembolization on pulmonary capacitance vessels and about the localization of the increased vascular resistance An increased pressure drop has been recorded between small pulmonary veins and left atrium after intravenous infusion of platelet aggregating substances such as ADP protamine and thrombin (Hyman *et al* 1971 Rådegran Drugge and Olsson 1972) This observation might indicate some active constriction of pulmonary veins Pulmonary venous hypertension and venous constriction have also been reported after lung embolism with autologous blood clots (Daicoff *et al* 1968) Venous constriction may again mean a reduction in lung vessel capacity even if the pulmonary veins may not dominate organ vascular capacity to the same extent as do venous vessels in organs of the systemic circulation (Fishman 1966)

There are also observations indicating that the volume of lung arteries may be altered upon intrapulmonary aggregation of blood platelets Subsequent to air embolization of the lungs Josephson and Ovenfors (1970) demonstrated that central pulmonary arteries became distended It is claimed that participation of blood platelets is essential to the pulmonary changes elicited by air emboli (Kahn *et al* 1972) These changes include increased vascular as well as transpulmonary pressure In isolated lungs an increased pulmonary blood volume has been found subsequent to such changes (Rosenzweig Hughes and Glazier 1970)

Detailed knowledge about the pulmonary vascular changes after intravascular platelet aggregation is wanted including the site and extent of capacitance alterations as well as the localization of the increased resistance Typical of the so-called shock lung is pulmonary microemboli congestion and interstitial edema (Blaisdell and Schlobohm 1973) Platelet aggregation and consumption seem to be an important event in the development of shock and pulmonary insufficiency after trauma (Blaisdell Lim and Stallone 1970 Ljungquist Bergentz and Lewis 1971) hypovolemia (Allardyce *et al* 1969 Bø and Hognestad 1971) anaphylaxis (Robb 1967) endotoxemia (Robb Margulis and Jabs 1972) and experimental disseminated intravascular coagulation (Radegran Swedenborg and Olsson 1972) Intrapulmonary blood platelet aggregation may also be a crucial event in connection with the circulatory and respiratory collapse after experimental pulmonary emboli (Thomas *et al* 1965 Bø Hognestad and Vaage 1974)

The main purpose of this investigation was to study the site and extent of the changes in pulmonary blood volume following sudden intravascular aggregation of blood platelets Besides we wanted to see if these changes were followed by changes in the extravascular water volume of the lungs

Methods

Cats *weighing* 2 7—3 7 *kg* were used *for* all experiments Anesthesia was introduced by light ether administration After venous catheterization chloralose and pentobarbitone sodium were injected intravenously 50 and ? mg/kg b wt respectively A tracheotomy was performed When the animals had been curarized by Alloferine® Hoffman-La Roche 0 1 ml/kg b wt.) positive pressure ventilation was started The frequency was 21/min and end expiratory *pressure was kept at* +1 5 cm H_2O The tidal volume was regulated to give an initial value of pH in arterial blood between 7.35 and 7 45 At 30 min interval inflation pressure was raised to 20 cm H_2O for one or two inflations

The thorax was widely opened by a midsternal incision Catheters could then be inserted into the pulmonary artery through the right ventricle and in the left atrium through its appendage Also one of the femoral arteries was cannulated and intravascular pressures were recorded on an eight-channel *polygraph* (*Grass Model 7B* Grass Instrument Co, Mass USA) through Statham pressure transducers (P23Gb P23Db and P23De) An electromagnetic flowprobe was mounted on the ascending aorta and connected to a Nycotron square wave flowmeter (type 37? Nycotron A/S Norway) Instantaneous and mean flow could then be recorded on the polygraph

Measurements of intra and extravascular fluid compartments in the lung A biopsy technique was used involving isotope labelling of blood and removal of isolated lung lobes The method has been described in detail previously (*Aarseth and Bø 19/2*) Silk snares were placed loosely around the hila of the two upper lung lobes and the right middle lobe When the blood was evenly labelled by added portions of ^{131}I human serum albumin and ^{51}Cr labelled erythrocytes the snares could be suddenly tightened and the lobe with its fluid content

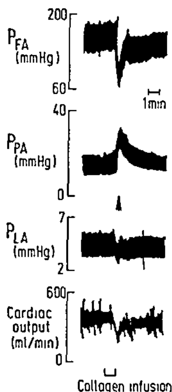


Fig 1

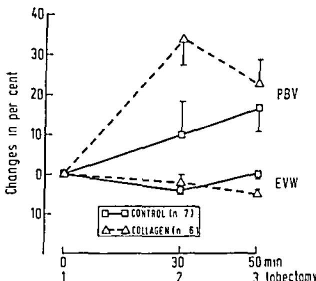


Fig 2

Fig 1 The effect of i v collagen infusion on the pulmonary circulation in an open-chested cat. Tracings from above femoral arterial pressure (P_{FA}) pulmonary arterial pressure (P_{PA}) left atrial pressure (P_{LA}) and cardiac output During the interval signalled at the bottom a collagen suspension was infused i v The arrow indicates the peak response the time at which the blood volume alterations were estimated

Fig 2 Pulmonary blood volume (PBV) and lung extravascular water volume (EVW) in two groups of cats The estimations have been performed by removing 3 lung lobes at zero time and after 30 and 50 min respectively The volumes found in the first lobe are used as reference values the ordinate gives the percentage changes in the second and third lobe To the animals in one group a collagen suspension has been infused i v prior to the removal of the second lobe Mean values Bars represent 1 S E

removed From the isotope-content of that lobe and in a simultaneously withdrawn arterial blood sample the plasma and erythrocyte volume of the lobe could be calculated From the wet and dry weight of the tissue, as well as of plasma and erythrocyte-samples also the extravascular water volume of that lobe was found All values were calculated relative to the dry net tissue weight of the lobe This allowed a comparison between the 3 different lobes that were removed from each animal

The right upper lobe was removed first, and used as a reference for that particular animal 30 min later 0 5 ml of a collagen suspension (collagen delivered by Sigma, prepared as described by Holmsen Day and Storm (1969)) was infused i v in the course of 1 min Such an infusion of collagen is followed by an increased pulmonary vascular resistance with a peak pulmonary arterial pressure after about 1 min At the top of the increase in pulmonary arterial pressure the circulation through the left upper lobe was stopped by tightening the silk snare and the lobe removed Twenty min later when vascular pressures were normal a third lobe was removed

TABLE I Pulmonary arterial pressure at the time of 3 separate lobectomies in cats The pressure at the first lobectomy is used as a reference in each animal (100) One group of cats received an intravenous infusion of collagen just prior to the removal of the second lobe The other group served as controls Mean values ± S D

	Pulmonary arterial pressure ( )	
	Collagen	Control
Lobe 1	100	100
Lobe 2	259±14	10?±7
Lobe 3	110±3	111±5

A similar procedure of lobe removal was performed in a control series of animals where there were no intervening infusions of collagen

Plasma osmolarity and plasma protein were measured in 2 expts at the time of lobe re movals The total plasma osmolarity was measured with a Knauer freeze-depression osmometer Protein content of plasma was found by spectrophotometric readings at 260 and 280 nm (Kalckar 1947)

Angiographic studies The surgical preparation of the 3 animals used for these experiments was as described above except that the left atrial catheter and the flow probe were not used here In stead in these animals a 30 cm long polyethylene catheter FG4 (Portex England) was inserted into the right external jugular vein and advanced into the superior caval vein Through this 1 ml/kg b wt of a contrast medium (Isopaque Cerebral® Nyco Norway) was injected by hand in the course of less than 1 s Serial films 2/s for 2 s and then 1/s for 6 s were obtained by using an Elema Scholander cut film changer and the following exposure data 800 mA 45 kV 0 0064 s film focus distance 100 cm and focal spot of the tube 1 2×1 2 mm Agfa Gevaert Curix RO? NIF films were used

When the surgical procedures were finished an injection of the contrast medium and serial filming were performed One hour later with the animal in the same position a collagen infusion was gi en and at peak pulmonary arterial pressure another injection of the contrast medium with serial filming was performed

Statistics Wilcoxon two-sample test was used in comparing the results from the test and control groups

Results

When a collagen suspension is infused as in the present experiments there is a subsequent constriction of pulmonary vascular as well as airway smooth muscle cells These responses which have been described in details previously can be re produced several times (Bo and Hognestad 1972 Vaage Bo and Hognestad 1974)

TABLE II Plasma protein content and plasma osmolarity at the time of 3 lobectomies in 2 cats (I and II) During intravascular platelet aggregation (lobe 2) there is possibly a small decrease in plasma protein content Afterwards there is a small increase in plasma osmolarity (lobe 3)

	Plasma protein content (mg/ml)		Plasma osmolarity (mosmol)	
Cat No	I	II	I	II
Lobe 1	61	73	3?1	315
Lobe 1	55	63	330	313
Lobe 3	54	69	339	3?3

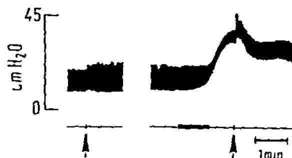


Fig 3 a. Effect on pulmonary arterial pressure of injection of standard dose of contrast medium. Injection of contrast medium into the superior caval vein carried out at arrow 3 b Same type of recording Collagen infused is during interval signalled at bottom At arrow the contrast medium was injected

The pulmonary arterial pressure increased cardiac output was reduced and the left atrial pressure was unchanged demonstrating an increased pulmonary vascular resistance The systemic arterial blood pressure was reduced in accordance with the reduced cardiac output. A typical response is shown in Fig 1 Concomitant with the pulmonary pressor responses there is a transient fall in the number of circulating platelets by about 30 per cent

In Fig 2 are shown how pulmonary blood content and lung extravascular water volume changed with time in the control animals and in the animals to which collagen was administered. The change in extravascular lung water was very small and insignificant at the time when the pressor response after collagen infusion was maximal ($p = 0.18$) However, 20 min later the lung water content in these animals was reduced as compared to the control group of animals ($p = 0.001$) At the time of maximal response to collagen there was an increased pulmonary blood volume ($p = 0.03$) 20 min later when the pressure effects of the collagen infusion had disappeared the lung blood volume was nearly normalized Table I demonstrates the corresponding changes in pulmonary arterial pressure

There were only small changes in plasma osmolarity and in plasma protein content during and after the collagen induced aggregation of blood platelets as shown in Table II

The angiographic studies were performed in order to get more information on the partition of the lung blood volume after platelet aggregation Injection of the contrast medium caused an increased pulmonary arterial pulse pressure (*Fig 3 a*) This effect was more marked when contrast was injected during the peak response after infusion of collagen (Fig 3 b)

Angiograms from a control injection of the contrast medium are shown in Fig 4 a and 4 b Fig 4 c and 4 d show corresponding angiograms taken during the peak pressor response after an infusion of collagen This infusion has a marked effect on both arteries and veins. The large arteries have increased their diameter On the other hand the peripheral arteries (diameter < 0.5 mm) do not seem to be dilated They are presented in the angiograms with an almost "cut off" appearance Contrary to the large arteries the large veins have a smaller diameter than in the control situation The small veins are too poorly filled with the contrast



Fig 4 a and b Pulmonary angiograms from cat Arterial and venous f lling respecti ely The arrows mark where vessel diameters have been measured 4 c and d Same animal pulmonary angiograms (with arterial and venous filling) at peak vascular response after i v collagen infusion For further information see Methods

TABLE III Mean of 3 measurements of diameters (in millimeters) in a large pulmonary artery (PA) and a large pulmonary vein (PV) before and after the i v administration of collagen Pulmonary circulation time (PCT) is given in seconds

Cat no	Before collagen			After collagen		
	PA	PV	PCT	PA	PV	PCT
I	3 6	2 7	1 5	3 9	2 6	2 5
II	5 4	4 5	2 0	7 3	3 6	3 5
II*	—	—	—	8 0	3 8	3 5
III	3 4	3 0	2 0	3 8	2 5	3 5
III*	3 6	2 6	2 0	3 9	2 6	3 5

* Repeated tests in the same animals 20 min after the first study For further details see test

medium to be measured properly In Table III are given the diameters of large arteries and veins before and after 5 collagen infusions Table III also gives the pulmonary circulation times which were measured as the time interval between appearance of the contrast medium in large pulmonary arteries and large pulmonary veins The increase in pulmonary circulation time after collagen infusion also shows up in the angiograms (4 a—4 d) It can be seen that there is a delayed emptying of the contrast medium from the arteries at the same time venous filling is reduced

Discussion

Pulmonary microembolization by platelet aggregates is known to induce an active constriction of pulmonary resistance vessels In the present series of experiments we wanted to focus the attention on the events in the consecutive segments of the pulmonary vasculature and the localization of resistance and capacitance changes after induced intravascular aggregation of blood platelets We particularly wanted to evaluate the volume changes occurring on the arterial and on the venous sides of the pulmonary capillaries By combining informations about resistance and volume changes with angiographic observations a more detailed description of the vascular events could be given Changes in extravascular water content would reflect changes in mean hydrostatic capillary pressure which is again determined by the ratio between pre and postcapillary resistance (Aarseth and Bo 1972)

Intravascular aggregation of blood platelets was achieved by i v infusions of collagen Several investigators have documented that the lungs have a filter function for platelets and platelet aggregates (Nordoy and Chandler 1964 Jenevein and Weiss 1964 Hauge Lunde and Waaler 1966 Ljungquist Bergentz and Lewis 1971) Histological studies after infusions of collagen reveal trapping of platelet aggregates in small arteries (< 100 μm) as well as in capillaries (Bo and Hognestad 1972)

Within a minute after collagen infusions the pulmonary vascular resistance increases tremendously An increased pulmonary blood volume and nearly unchanged lung extravascular water volume were revealed in the lobes removed during the peak of the pressor response after collagen infusion (Fig 2) The partition of the organ blood volume is unique in the lungs Here the arterial resistance vessels accommodate almost as large a volume as do the traditional venous capacitance vessels (Fishman 1966) It may therefore be surprising to observe that both the resistance and the capacitance of the pulmonary vascular bed can be increased simultaneously This finding is all the more puzzling as some narrowing of veins has been suggested to take place during intravascular platelet aggregation (Hyman *et al* 1971 Radegran Drugge and Olsson 1972) However the finding of an unchanged lung water content combined with a major increase in pulmonary arterial pressure is consistent with a powerful precapillary constriction If a larger part of this pressure increase was allowed to act on the pulmonary capillaries it would be expected to affect the transvascular fluid balance profoundly Minor changes however might not be detected in the present experiments since the pressure in the pulmonary artery had been increased for only about one minute Thus a smaller postcapillary constriction cannot be excluded In fact there was a small but insignificant increase in lung water after induction of intravascular platelet aggregation (Fig 2)

An interesting question is how the changes in airway pressure during intravascular platelet aggregation may affect the transvascular fluid balance in the lungs Usually an increased transpulmonary pressure as seen after platelet microemboli (Vaage Bo and Hognestad 1974) is expected to reduce lung water volume (Sugerman *et al* 1972) However after infusion of collagen the increased transpulmonary pressure is caused by narrowing and closure of peripheral airyays Collapse of peripheral airways has been shown to increase extravascular lung water content (Bo and Vaage 1973) One can therefore not postulate that the airway changes will greatly counteract any vascular changes tending to increase lung water volume

The angiographic studies of the present experiments revealed a marked dilatation of the larger pulmonary arteries on top of the pressor responses The largest effect was on the main branches which are predominantly of the elastic type The cut off" appearance of the smallest arteries visible suggests on the other hand an increased tone in precapillary vessels Simultaneously the pulmonary veins have a reduced diameter These findings explain how pulmonary blood content can be increased without venous congestion and also how there is simultaneously a rise in vascular resistance A precapillary constriction elevates pulmonary arterial pressure to such an extent that passive distension of the large elastic pulmonary arteries takes place inducing a shift of blood from the venous to the arterial compartment of the pulmonary vascular bed

The reduced venous diameter is difficult to interpret It may be due to an active constriction of the veins but passive recoil caused by reduced blood flow is an equally likely explanation An increased pressure difference along the pulmonary

veins as reported by other investigators (Hyman *et al* 1971 Rådegran Drugge and Olsson 1972) does not *per se* indicate an active vasoconstriction in this segment Besides the drop in total flow also the flow pattern through the lungs may be changed subsequent to platelet microemboli Such changes will include obstruction of some microvessels with an uneven perfusion of the lung tissue Some vessels might indeed have an increased flow, thereby increasing pressure in downstream vessels while other parts of the microcirculation are more or less obstructed, thus decreasing downstream flow and pressures (Hyman 1969 a) In this connection it is interesting to note that anatomical arteriovenous shunts have been said to open up after lung emboli (Niden and Aviads 1956) and in shock lung patients (Germon Kazun and Brady 1968) Unsystematical venous pressure changes have recently been demonstrated after embolization with blood clots in an hemodynamically isolated lung lobe perfused with constant flow (Woolverton and Hyman 1973) Using two catheters in different lobar veins the pressure was observed to increase in one vein while it decreased in the other vein Possibly a catheter in a small pulmonary vein might get into a wedge position because of passive *recoil if flow in* that particular vein is reduced In this way a false high pressure might be measured However serotonin and prostaglandin F_2 which both are among the possible mediators of the pulmonary pressor responses during intravascular platelet aggregation (Daicoff *et al* 1968 Thomas *et al* 1965 Piper and Vaage 1974) are reported to constrict pulmonary veins (Daicoff *et al* 1968 Hyman 1969 b 1969 c) In conclusion the question of active pulmonary venoconstriction in intravascular platelet aggregation is not solved

At the time of the third lobectomy, 20 min after collagen infusion the vascular pressure response had disappeared As shown in Fig 2 the lung blood volume was also close to normal in this situation suggesting a common denominator for pressure and volume changes At this time however there is a significantly reduced extravascular water volume One explanation of this finding could be if arterial as well as venous vasoconstriction are induced by humoral mechanisms during lodging of platelet aggregates in small arteries it is conceivable that the most remote effects (that on the veins) would disappear first As the exposure of vasoactive substances is getting smaller the ratio between pre and postcapillary resistance may then change the postcapillary resistance increase tailing off first This would intermittently leave a reduced mean hydrostatic capillary pressure with reduced extravascular water content In addition an increased plasma osmolarity seems to be present at the time of the third lobectomy (Table II) Such a change is also known to reduce extravascular lung water content (Aarseth Bo and Piene 1973) Besides some microvessels may still be partly or completely obstructed by platelet thrombi Consequently small segments of the lung may be subjected to hypoperfusion or to a lowered hydrostatic capillary pressure *due to prolonged mechanical* obstruction

The conclusion from the present experiments on intravascular platelet aggregation is that the active event is primarily a constriction of the smaller arterial vessels

with increased precapillary resistance The increase of pulmonary blood volume and the shift of blood from the venous to the arterial part of the pulmonary vascular bed appear to be secondary passive effects due to a dilatation of the large pulmonary arteries Venous narrowing might be caused by active constriction by passive mechanical changes or by a combination of these two factors

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References

AARSETH P and G Bo Content of blood and of extravascular water in cat lungs during changes in total blood volume. *Acta physiol scand* 1972 *85* 343—352

AARSETH P G Bo and H PIENE Plasma hyperosmolarity and pulmonary fluid compartments *Acta physiol scand* 1973 Suppl 396 62

ALLARDYCE B H F HAMIT T MATSUMOTO and R V MOSELEY Pulmonary vascular changes in hypovolemic shock Radiography of the pulmonary microcirculation and the possibl role of platelet embolism in increasing vascular resistance *J Trauma* 1969 *9* 403—411

ARFORS K E C BUSCH S JAKOBSON O LINDQUIST P MALMBERG L RAMMER and T SALDEEN Pulmonary insufficiency following intravenous infusion of thrombin and AMCA (tranexamic acid) in the dog *Acta chir scand* 1972 *138* 445—5?

BERMAN I R M E SMULSON P PATTENGALE and S F SCHOENBACH Pulmonary microembolism after soft tissue injury in primates *Surgery* 1971 *70* 246—52

BLAISDELL F W R C LIM and R J STALLONE The mechanism of pulmonary damage following traumatic shock *Surg Gynec Obstet* 1970 *130* 15—2?

BLAISDELL F W and R M SCHLOBOHM The respiratory distress syndrome A review *Surgery* 1973 *74* 251—62

Bo G and J HOGNESTAD Thrombocytes and pulmonary vascular resistance during hemorrhagic hypotension *Acta physiol scand* 1971 *82* ?18—?8

Bo G and J HOGNESTAD Effect on the pulmonary circulation of suddenly nduced intravascular aggregation of blood platelets *Acta physiol scand* 1972 *85* 5?3—31

Bo G J HOGNESTAD and J VAAGE The role of blood platelets in pulmonary responses to microembolization with barium sulphate *Acta physiol scand* 1974 *90* 244—251

Bo G and J VAAGE *Lung water vol me in atelectasis* 7th Europ Conf Microcirculation Aberdeen 1972 part II B bl anat (Karger Basel) 1973 No 12 pp 376—80

DAICOFF G R F R CHAVEZ A H ANTON and E W SWENSON Serotonin induced pulmonary venous hypertension in pulmonary embolism *J thorac cardiovasc Surg* 1968 *56* 810—16

FISHMAN A P The volume of blood in the lungs *Circulat on* 1966 *33* 835—838

GERMON P A I KAZUN and L W BRADY Shunting following trauma. *J Trauma* 1968 *8* 724—34

HALGE A P K M LUNDE and B A WAALER, Some observations on trapping and release of thrombocytes in isolated perfused rabbit lungs In *Conference on pulmonary circulation* Oslo 1965 Ed Carsten Muller Scandinavian University Books Universitetsforlaget, O lo 1966 pp 83—93

HOLMSEN H H J DAY and E STORM Aden ne nucleotide metabolism of blood platelets VI Subcellular localization of nucleotide pools with different functions in the platelet release reaction *Bioch m biophys Acta* (Amst.) 1969 *186* ?54—66

HYMAN A L Effects of large increases in pulmonary blood flo v on pulmonary venous pressure *J appl Physiol* 1969 a ?7 179—185

HYMAN A L The direct effects of asoactive agents on pulmonary veins Stud es of responses to acet lchol ne serotonin histam ne and isoproterenol in intact dogs *J Pharmacol exp The* 1969 b *168* 96—105

HYMAN A L The acti e responses of pulmonary veins in intact dogs to prostaglandins F2 alpha and E1 *J Pharmacol exp Ther* 1969 c *165* 267—73

HYMAN A L W C WOOLVERTON D G PENNINGTON and W E JAQUES Pulmonary vascular resp nse to adenosine diphosphate *J Pharmacol xp The* 1971 *178* 549—61

JENEVEIN E P and D L WEISS Platelet m croemboli associated with massive blood transfusion *Ame J Path* 1964 *45* 313—?1

JOSEPHSON S and C O OVENFORS Experimental pulmonary air-embolism An angiographic study in the dog *Invest Radiology* 1970 *5* 220—31

KALCKAR, H M Differential spectrophotometry of purine compounds by means of specific enzymes III Studies of the enzymes of purine metabolism *J biol Chem* 1947 *167* 461—475

KHAN M A I ALKALAY S SUETSUGU and M STEIN Acute changes in lung mechanics following pulmonary emboli of various gases in dogs *J appl Physiol* 1972 *33* 774—77

LJUNGQUIST U S E BERGENTZ and D H LEWIS The distribution of platelets fibrin and erythrocytes in various organs following experimental trauma. *Europ Surg Res* 1971 *3* 293—300

NIDEN A H and D M AVIADO Effects of pulmonary embolism on the pulmonary circulation with special reference to arteriovenous shunts in the lung *Circulat Res* 1956 *4* 67—73

NORDOY A. and A. B CHANDLER Platelet thrombosis induced by adenosine diphosphate in the rat. *Scand J Haemat* 1964 *1* 16—25

PIPER, P J and J VAAGE Platelet aggregation pulmonary microembolism and the release of prostaglandins 1974 *In preparation*

RÅDEGRAN K L DRUGGE and P OLSSON *Pulmonary venoconstriction by induced platelet aggregation VII Conference on Microcirculation European Society for Microcirculation* Aberdeen 1972

RADEGRAN K and C MCASLAN Circulatory and ventilatory effects of induced platelet aggregation and their inhibition by acetylsalicylic acid *Acta anaesth scand* 1972 *16* 76—84

RÅDEGRAN K J SWEDENBORG and P OLSSON Effect of defibrinogenation and acetylsalicylic acid on the circulatory response to thrombin *Acta chir scand* 1972 *138* 441—44

ROBB H J Observations on dynamics of microcirculation during experimental anaphylaxis *Ann Allergy* 1967 *25* 78—86

ROBB H J R. R. MARGULIS and C M JABS Role of pulmonary microembolism in the hemodynamics of endotoxin shock *Surg Gynec Obstet* 1972 *135* 777—83

ROSENZWEIG D Y J M B HUGHES and J B GLAZIER Effects of transpulmonary and vascular pressures on pulmonary blood volume in isolated lung *J appl Physiol* 1970 *28* 553—60

SUGERMAN H J K. B OLOFSSON T W POLLOCK R F AGNEW R. M ROGERS and L D MILLER Continuous positive end-expiratory pressure ventilation (PEEP) for the treatment of diffuse interstitial pulmonary edema. *J Trauma* 1972 *12* 263—73

THOMAS D P G TANABE M KHAN and M STEIN Humoral factors mediated by platelets in experimental pulmonary embolism. In *Pulmonary emboli disease* Ed A A Sasahara and M Stein New York and London Grune and Stratton 1965 pp 59—64

VAAGE J G BO and J HOGNESTAD Pulmonary responses to repeatedly induced intravascular platelet aggregation. *Microvasc Res* 1973 *6* 257

VAAGE J G BO and J HOGNESTAD Pulmonary responses to intravascular platelet aggregation *Acta physiol scand* 1974 in press

WOOLVERTON W C and A L HYMAN The pulmonary hemodynamic effects of lung thromboemboli in dogs *Surgery* 1973 *73* 572—78

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From the Institute of Physiology University of Oslo Norway

Release of Kallikrein from Isolated Cellular Organelles of the Rat Submandibular Salivary Gland

By

K. M Gautvik and M Kriz

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Abstract

Gautvik K. M and M Kriz *Release of kallikrein from isolated cellular organelles of the rat submandibular salivary gland* Acta physiol scand 1974 92 95—102

Subcellular organelles which contained kallikrein were isolated from homogenates of rat submandibular salivary glands by sucrose density gradient centrifugation Kallikrein activity and BAEE-esterase activity were localized to the same subcellular fractions The BAEE-esterase containing organelles lysed easily in normoosmolar buffer solutions and liberated soluble enzyme The spontaneous lysis of these organelles was reduced in hyperosmolar solutions The presence of calcium (2 6—27 mM) stabilized the enzyme containing organelles but magnesium (25 mM) and potassium (60 mM) had no such effect. Replacement of sodium with sucrose and removal of calcium magnesium or potassium had also no effect on enzyme release

Kallikreins are enzymes which cause specific and rapid formation of kinins from certain plasma proteins kininogens but show no or only a small proteolytical activity towards other substrates such as casein or hemoglobin (Webster 1970) One group of kallikreins is found in exocrine glands and in their secretions Various types of evidence suggest thtat these enzymes play a role in the regulation of blood flow in salivary glands (Hilton and Lewis 1956 Gautvik 1970)

Knowledge of the subcellular distribution of kallikrein and of factors which affect its release may lead to a better understanding of the physiological role of this enzyme

The present work is an analysis of how various changes in the surrounding medium affect the liberation of kallikrein from isolated subcellular salivary gland organelles

Materials and methods

Isolation of kallikrein containing organelles 20—30 rats of both sexes were used for one series of experiments The submandibular salivary glands were removed immediately after decapitation of the animals and washed in ice-cold 0 7 M sucrose The glands were then cleaned dried weighed and cut into small pieces. The tissue was homogenized for 3 min with a Dounce homogenizer in a volume of 0 7 M sucrose (10× the wet weight)

JOSEPHSON S and C O OVENFORS Experimental pulmonary air embolism An angiographic study in the dog *Invest Radiology* 1970 5 220—31

KALCKAR H M Differential spectrophotometry of purine compounds by means of specific enzymes III Studies of the enzymes of purine metabolism *J biol Chem* 1947 167 461—475

KHAN M A I ALKALAY S SUETSUGU and M STEIN Acute changes in lung mechanics following pulmonary emboli of various gases in dogs *J appl Physiol* 1972 33 774—77

LJUNGQUIST U S E BERGENTZ and D H LEWIS The distribution of platelets fibrin and erythrocytes in various organs following experimental trauma *Europ Surg Res* 1971 3 293—300

NIDEN A H and D M AVIADO Effects om pulmonary embolism on the pulmonary circulation with special reference to arteriovenous shunts in the lung *Circulat Res* 1956 4 67—73

NORDOY A and A B CHANDLER Platelet thrombosis induced by adenosine disphosphate in the rat *Scand J Haemat* 1964 1 16—25

PIPER P J and J VAAGE Platelet aggregation pulmonary microembolism and the release of prostaglandins 1974 In preparation

RÅDEGRAN K U DRUGGE and P OLSSON *Pulmonary venoconstriction by induced platelet aggregation* VII Conference on Microcirculation European Society for Microcirculation Aberdeen 1972

RADEGRAN K and C MCASLAN Circulatory and ventilatory effects of induced platelet aggregation and their inhibition by acetylsalicyclic acid *Acta anaesth scand* 1972 16 76—81

RÅDEGRAN K J SWEDENBORG and P OLSSON Effect of defibrinogenation and acetylsalicylic acid on the circulatory response to thrombin *Acta chir scand* 1972 138 441—44

ROBB H J Observations on dynamics of microcirculation during experimental anaphylaxis *Ann Allergy* 1967 25 78—86

ROBB H J R R MARGULIS and C M JABS Role of pulmonary microembolism in the hemodynamics of endotoxin shock *Surg Gynec Obstet* 1972 135 777—83

ROSENZWEIG D Y J M B HUGHES and J B GLAZIER Effects of transpulmonary and vascular pressures on pulmonary blood volume in isolated lung *J appl Physiol* 1970 28 553—60

SUGERMAN H J K B OLOFSON T W POLLOCK R F AGNEW R M ROGERS and L D MILLER Continuous positive end expiratory pressure ventilation (PEEP) for the treatment of diffuse interstitial pulmonary edema *J Trauma* 1972 12 263—73

THOMAS D P G TANABE M KHAN and M STEIN Humoral factors mediated by platelets in experimental pulmonary embolism In *Pulmonary embolic disease* Ed A A Sasahara and M Stein New York and London Grune and Stratton, 1965 pp 59—64

VAAGE J G BO and J HOGNESTAD Pulmonary responses to repeatedly induced intravascular platelet aggregation *Microvasc Res* 1973 6 257

VAAGE J G BO and J HOGNESTAD Pulmonary responses to intravascular platelet aggregation *Acta physiol scand* 1974 in press

WOOLVERTON W C and A L HYMAN The pulmonary hemodynamic effects of lung thromboemboli in dogs *Surgery* 1973 73 572—78

Acta physiol scand 1974 92 95—102

From the Institute of Physiology University of Oslo Norway

Release of Kallikrein from Isolated Cellular Organelles of the Rat Submandibular Salivary Gland

By

K M Gautvik and M Kriz

Received 28 February 1974

Abstract

Gautvik K M and M Kriz *Pelease of kallikrein from isolated cellular organelles of the rat submandibular salivary gland* Acta physiol scand 1974 92 95—102

Subcellular organelles which contained kallikrein were isolated from homogenates of rat submand bular salivary glands by sucrose density gradient centrifugation Kallikrein activity and BAEE-esterase activity were localized to the same subcellular fractions The BAEE-esterase containing organelles lysed easily in normoosmolar buffer solutions and liberated soluble enzyme The spontaneous lysis of these organelles was reduced in hyperosmolar solutions The presence of calcium (2 6—27 mM) stabilized the enzyme containing organelles but magnesium (25 mM) and potassium (60 mM) had no such eff ct Replacement of sodium with sucrose and removal of calcium magnesium or potassium had also no effect on enzyme release

Kallikreins are enzymes which cause specific and rapid formation of kinins from certain plasma proteins kininogens but show no or only a small proteolytical activity towards other substrates such as casein or hemoglobin (Webster 1970) One group of kallikreins is found in exocrine glands and in their secretions Various types of evidence suggest thtat these enzymes play a role in the regulation of blood flow in salivary glands (Hilton and Lewis 1956 Gautvik 1970)

Knowledge of the subcellular distribution of kallikrein and of factors which affect its release may lead to a better understanding of the physiological role of this enzyme

The present work is an analysis of how various changes in the surrounding medium affect the liberation of kallikrein from isolated subcellular salivary gland organelles

Materials and methods

Isolation of kallikrein containing organelles 20—30 rats of both sexes were used for one series of experiments The submandibular salivary glands were removed immediately after decapitation of the animals and washed in ice-cold 0 7 M sucrose The glands were then cleaned dried weighed and cut into small pieces. The tissue was homogenized for 3 min with a Dounce homogenizer in a volume of 0 7 M sucrose (10× the wet weight)

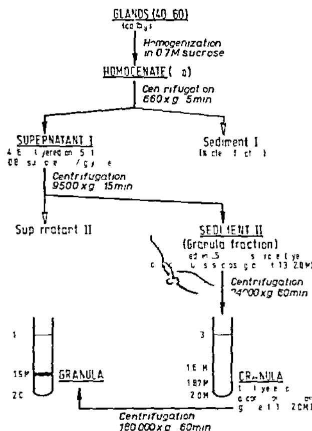


Fig. 1. Schematical diagram of the procedure for homogenization of rat submandibular salivary glands and isolation of the kallikrein and esterase containing cellular organelles.

The homogenate was centrifuged at 660×*g* for 5 min at +18° C. 4—6 ml of the supernatant was layered on top of 1.5 ml 0.8 M sucrose containing 1 % glycogen and processed as described in Fig. 1. The cellular organelles containing kallikrein and esterase activity were recovered after the last centrifugation in sucrose fraction of 1.9 M.

Measurements of enzymes. Rat salivary kallikrein was measured through its kinin formation on the isolated rat uterus preparation as described previously (Gautvik, Nustad and Vystad 1972). The enzyme activity was expressed as bradykinin equivalents (µg bradykinin/mg protein).

Esterase activity in kallikrein containing organelles was also determined using a synthetic substrate: α-N-Benzoyl-L-Arginine Ethyl Ester HCl (BAEE) (Gautvik, Nustad and Vystad 1972). The enzyme activity was expressed as µM BAEE hydrolyzed per min and mg protein.

Acid phosphatase was measured by incubating 0.2 ml of enzyme solution with 0.5 ml of 0.4 ... disodium p-nitro-phenyl orthophosphate (Sigma) in the presence of 0.5 ml 0.09 M citrate buffer pH 4.8 as described by Sigma, Technical Bulletin No. 104 March 1973.

Cytochrome oxidase was determined as described by Cooperstein and Lazarow (1951). To 3 ml of reduced cytochrome C (Sigma) (1.7×10⁻⁵ M in 0.03 M phosphate buffer containing 0.04 M $Na_2S_2O_4$) was added 0.02 ml to 0.04 ml of enzyme solution. The oxidation of reduced enzyme was read every 30 s at 550 nm in a Zeiss spectrophotometer.

Protein was determined by the method of Kalckar (1947).

Results

Isolation of kallikrein containing organelles. Kallikrein has earlier been shown to be localized in subcellular organelles and electronmicroscopic studies have revealed that they resemble zymogen granules (Erdos, Tague and Miwa 1968; Bhoola 1969

TABLE I Enzyme activities in subcellular fractions*

	Cytochrome oxidase	Acid phosphatase	BAEE esterase	Kallikrein	Protein (mg/ml)
Homogenate	100	100	100	100	700
Supernatant I (660 × g)	—	—	48.8	43.8	24
Supernatant II (9500 × g)	—	—	5.6	5.6	15
Sediment (9500 × g)	47	78	96	100	14
Granule fraction (1.3M–2.0M)	28	111	193	215	0.65
Granule fraction (1.7M–2.0M)	0	0	290	304	0.19

* Enzyme activities were calculated per mg protein. The enzyme concentration in homogenate was set equal to 100 and the specific activities calculated accordingly. For details see Methods.

Bhoola and Heap 1970). The present procedure used for isolation of the rat secretory granules is presented in a schematic flow diagram shown in Fig. 1. This procedure is that described by Bhoola and Heap (1970) with some modifications.

In the final fraction isolated after the second sucrose density gradient centrifugation, the specific activity of kallikrein was increased about 3 fold, while cytochrome oxidase and acid phosphatase activity were no longer detectable (Table I).

The amount of kallikrein which was recovered after the second sucrose density gradient centrifugation was too small to be used for all the present experiments. Therefore, the organelle fraction isolated after the first sucrose density gradient centrifugation was generally used, although they contained cytochrome oxidase and acid phosphatase (Table I).

The kallikrein and the BAEE esterase activity changed in parallel during the procedures (Table I). As shown earlier by Erdös *et al.* (1968) and as later confirmed by Nustad and Gautvik (unpublished), most of the BAEE esterase activity in the rat submandibular gland can be accounted for by enzymes with kallikrein activity. Assay of BAEE esterase activity was therefore used as a measure for kallikrein in the following experiments (see Methods).

The effect of various buffers, osmolarity, D_2O and pH on the release of BAEE esterase activity from isolated subcellular organelles

Fig. 2 shows the effect of normo- and hyperosmolar solutions of Krebs-Ringer Tris buffer and of sucrose. Sodium chloride was added to the Krebs Ringer solution to give the desired hyperosmolarity. In normoosmolar solutions of Krebs-Ringer and of sucrose only about 10—20 % of the granules remained intact after incubation for 5 min at 37° C. The major part of the BAEE esterase activity was under these conditions recovered in the supernatant fraction (Fig. 2). However, the kallikrein containing organelles were much more stable in hyperosmolar solution where not more than 50 % of the enzyme activity was found in the supernatant fraction after a 5 min incubation (Fig. 2). Other buffer systems of similar osmolarity and with

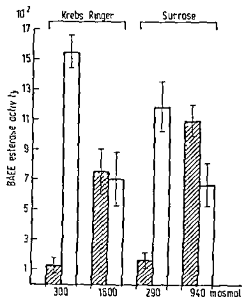


Fig 2

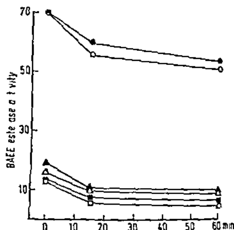


Fig 3

Fig 2 Effect of normo- and hyperosmolar solutions on the liberation of esterase from isolated cellular organelles The enzyme containing organelles were resuspended in Krebs Ringer solution (phosphate replaced by Tris buffer) and sucrose of the osmolarities indicated below The samples were incubated at 37 C for 5 min and then centrifuged as described in Methods and the supernatant (soluble enzyme) and the sediment (intact granules) assayed for BAEE esterase activity The results are given in BAEE esterase units (see Methods) and represent mean values ±S E of 4 to 6 expts ▨ intact granules ☐ ruptured granules

Fig 3 Effect of deuterium oxide on esterase release from isolated cellular organelles The granule fraction was resuspended in solutions of the compositions shown below and incubated for the time periods given at 37 C The lysis of kallikrein containing particles was assayed by measuring the BAEE esterase activity remaining in the sedimentable fraction after the incubation Each point represents mean values of triplicate samples ● sucrose in H_2O (1300 mosmol) ○ sucrose in D O (1300 mosmol) ▲ sucrose in H O (290 mosmol) △ sucrose in D O (290 mosmol) ■ Krebs Ringer Tris in H O (300 mosmol) ☐ Krebs-Ringer Tris in D O (300 mosmol)

normal pH gave similar results The lysis of enzyme containing organelles was reduced by incubation both in normo and in hyperosmolar solutions when the temperature was kept at 20° C instead of 37° C (data not shown)

It has been reported that deuterium oxide is able to inhibit thyroid gland secretion (Williams and Wolff 1972) This chemical does probably affect microfilaments which are reported to be of importance for the cellular translocation of secretory granules to the cell surface during the secretory process (Williams and Wolff 1972) In order to test whether D O also has a direct effect on isolated secretory granules the kallikrein containing organelles were put into hyper and normoosmolar solutions made up in D O The release of enzyme into the supernatant was followed and compared with controls where the buffer and sucrose solutions were made up in H O As shown in Fig 3 the spontaneous liberation of enzyme from sedimental particles is very similar in the two situations There is again an organelle stal during

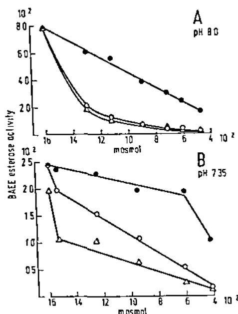


Fig 4

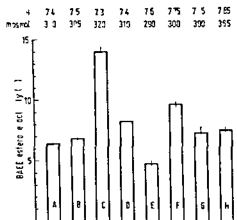


Fig 5

Fig 4 Spontaneous release of esterase from isolated granules incubated at pH 8 (A) and pH 7 35 (B) at 37 C for the time periods indicated below The granule fractions were incubated in Krebs Ringer solution (phosphate replaced by Tris) to which sucrose was added to give the desired osmolarity The results are given as BAEE esterase activity remaining in sedimentable particles after the incubation and each point represents mean values of 3 expts Incubation period ● 0 min ○ 15 min △ 60 min

Fig 5 Effect of calcium magnesium potassium and sodium on esterase release from cellular organelles The granule fractions were resuspended in Krebs Ringer solution (phosphate replaced by Tris) of the composition indicated below and incubated for 15 min for 37 C Enzyme activity remaining in intact granules was determined and the results expressed as per cent of total enzyme activity Mean values and ranges of two different experiments are shown pH and osmolarity are also given for each different group of samples A Normal Krebs Ringer *solution (phosphate replaced by Tris)* B *Calcium replaced by sodium.* C *27 mM of calcium* with an equimolar reduction in sodium D Potassium replaced by sodium E 60 mM potassium with an equimolar reduction in sodium F Magnesium replaced by sodium G 25 mM magnesium with equimolar reduction in sodium. H Sodium replaced by sucrose

effect of hyperosmolar sucrose but the presence of D O does not affect the spontaneous liberation of esterase activity

In Fig 4 is shown the time course of liberation of esterase at two different values of pH The kallikrein containing organelles were incubated at 37° C for the time periods shown in sucrose solution of different osmolarity There is clearly an increased rate of enzyme liberation at pH 8 at all time periods tested The difference is greatest for slight hyperosmolar solutions

Effects of cations and of ATP on the esterase release When the granules were incubated in normoosmolar Krebs Ringer Tris buffer for 15 min at 37° C about 6 % of the total BAEE esterase activity remained in the sedimental fraction (Fig 5 A) Removal of added calcium had no effect (Fig 5 B) but increasing the

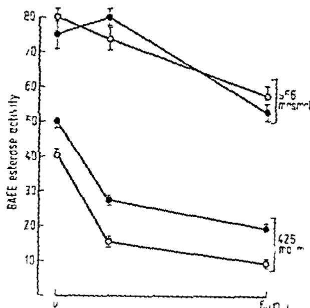


Fig. 6

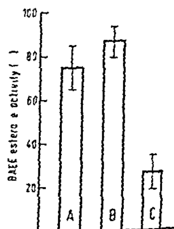


Fig. 7

Fig. 6. Effect of calcium on the release of esterase from cellular organelles resuspended in solutions of different osmolarity. The granule fractions were resuspended in Krebs-Ringer solution (phosphate replaced by Tris) with (●) or without (O) 2.6 mM calcium. Sucrose had been added to give the indicated osmolarities. Enzyme activity remaining in intact granules was determined after incubation at 37° C for the time periods shown. The results are given as mean ± S.E. of 4 samples.

Fig. 7. Effect of 1 mM ATP on the esterase containing granules. The granule fractions were resuspended in sucrose or Krebs-Ringer solution of different osmolarity with or without the addition of 1 mM ATP. The enzyme activity remaining in intact granules was measured after incubation for 15 min at 37° C. The enzyme activity in the control groups was set equal to 100 and the esterase activity in the ATP treated groups calculated accordingly. The results are expressed as mean ± S.E. of triplicates. A. Krebs-Ringer Tris 300 mosmol. B. Sucrose 940 mosmol. C. Sucrose 340 mosmol.

calcium concentration to 27 mM with preservation of normoosmolarity reduced the spontaneous lysis of the organelles (Fig. 5 C). However magnesium at 25 mM did not show this effect (Fig. 5 G). Removal of potassium, magnesium or sodium (sucrose replacing sodium chloride) had also no effect on the spontaneous liberation of enzyme from the granules (Fig. 5 D, F, H). The addition of potassium had a small, if any, stimulating effect on enzyme release (Fig. 5 F).

The stabilizing effect of 2.6 mM of calcium on the kallikrein containing organelles could be demonstrated up to 60 min of incubation in slight hyperosmolar solutions of Krebs-Ringer Tris (Fig. 6). In very hyperosmolar solutions (966 mosmol) the spontaneous liberation of kallikrein from granules was again reduced, but then the presence of calcium had no additional effect on the enzyme release.

Granule fractions incubated with sucrose of 340 mosmol for 15 min at 37° C in the presence of 1 mM ATP showed a greatly increased liberation of enzyme in com-

parison with controls which contained no ATP (Fig 7 C) In sucrose solution of 940 mosmol however ATP caused a much smaller stimulation of esterase release (Fig 7 B) In normoosmolar Krebs Ringer Tris solution 1 mM ATP also showed a small but significant stimulation of the release of esterase (Fig 7 A)

Discussion

This study has shown that it is possible to isolate a fraction of cellular organelles which contained a high specific activity of kallikrein but was devoid of any detectable cytochrome oxidase or acid phosphatase However the final purification step gave a small total yield of kallikrein Most of the subsequent experiments were therefore carried out with a subcellular fraction which also contained cytochrome oxidase and acid phosphatase enzymes which are used as markers for mitochondrial and lysosomal membranes respectively (Duve *et al* 1955) However the presence of other subcellular fractions would presumably not be important for the study of physio-chemical behaviour of these subcellular granules containing kallikrein

This study has clearly shown that the kallikrein containing and esterase containing particles were unstable in several of the usual laboratory buffers or in sucrose of normal osmolarity Hyperosmolar solutions did however reduce the spontaneous liberation of enzyme Also these organelles were more stable at normal than at high pH The physiological significance of these observations is uncertain This knowledge however is of practical importance for further work on isolated kallikrein containing granules

Secretion of kallikrein from salivary gland in response to activation of secretory nerves is accompanied by alterations in cell membrane potentials and in the intracellular concentration of certain cations (Pedersen 1972) It was of interest therefore to test the effect of some of these cations on the liberation of kallikrein (measured as esterase) from isolated subcellular organelles The main finding was that calcium stabilized the granules and that this effect was observed with the granules suspended in normo- or slight hyperosmolar solutions The effect is specific for calcium since similar concentrations of magnesium did not alter the spontaneous release of this enzyme Calcium is probably a key mediator of enzyme and hormone release in most secretory cells (Rasmussen 1970) One unsolved problem is how intracellular calcium affects the extrusion of the secretory granules from the cells It has been shown that calcium bind to isolated secretory granules and that this uptake of calcium fascilitates the fusion of the granules with the cell membrane a step which is a prerequisite for exocytic secretion (Dean personal communication) Calcium is also involved in the secretion of catecholamines (Douglas 1968) and has been reported to stimulate the release of catecholamines from isolated granules in the presence of phosphate but to have no such effect in solutions of sucrose (Lishajko 1970) In this study Tris replaced phosphate in Krebs Ringer solution because of the frequent precipitation of calcium phosphate whose mechanical effect on the intact granules was undesired Therefore the combined effect of calcium and

phosphate was not tested in this study Calcium alone had early no enzyme releasing effect when the granules were suspended in sucrose On the contrary, calcium then stabilized the granules (Fig 6) Calcium may thus affect directly the kallikrein containing granules also in the intact cell and this effect may be of importance for the ultimate release of the enzyme

ATP was able to stimulate the release of esterase in slight hyperosmolar solutions of sucrose The stimulatory effect of ATP was much less in very hyperosmolar solutions of sucrose or normoosmolar solutions of Krebs Ringer Tris In the latter situation an enhanced lysis of granules was difficult to detect due to the already high rate of spontaneous enzyme release The relationship between calcium and ATP if any in the secretory process is however, unknown

References

Bhoola K D Intracellular distribution of submaxillary kallikrein *J Physiol* (Lond) 1968 *196* 431—445

Bhoola K D and P F Heap Properties of kallikrein containing granules isolated from the submaxillary gland of the guinea pig *J Physiol* (Lond) 1970 *210* 421—432

Cooperstein S J and A Lazarow A microspectrophotometric method for the determination of cytochrome oxidase *J biol Chem* 1951 *189* 665—670

Douglas W W Stimulus secretion coupling the concept and clues from chromaffin and other cells. *Brit J Pharmacol* 1968 *34* 451—474

Duve de C B C Pressman R Gianetto R Wattiaux and F Appelmans *Tissue fractionations studies* 6 intracellular distribution patterns of enzymes in rat liver tissue *Biochem J* 1955 *60* 604—617

Erdös E G L L Tague and I Miwa Kallikrein in granules of the submaxillary gland *Biochem Pharmacol* 1968 *17* 667—674

Gautvik K M Studies on kinin formation in functional vasodilatation of the submandibular salivary gland in cats *Acta physiol scand* 1970 *79* 174—187

Gautvik K M K Nustad and J Vystyd Kininogenase in the stimulated submandibular salivary gland in cats *Acta physiol scand* 1972 *85* 438—443

Hilton S M and G P Lewis The relationship between glandular activity bradykinin formation and functional vasodilatation in the submandibular salivary gland *J Physiol* Lond 1956 *134* 471—483

Kalckar H M Differential spectrophotometry of purine compounds by means of specific enzymes *J biol Chem* 1947 *167* 461—475

Lishajko F Effect of calcium phosphate and RNA on the release of catecholamines ATP and protein from isolated adrenal medullary granules *Life Sci* 1970 9 695—699

Pedersen O H Acetylcholine induced ion transports involved in the formation of saliva *Acta physiol scand* 1972 Suppl 381

Rasmussen H Cell communication calcium ion and cyclic adenosine monophosphate *Science* 1970 *170* 404—412

Webster M E Kallikreins in glandular tissues In *Handbuch der experimentellen Pharmakologie* 1970 25 pp 131—155 Springer Berlin

Williams J A and J Wolff Colchicine binding protein and the secretion of thyroid hormone *J Cell Biol* 1972 *54* 157—165

Acta physiol scand 1974 92 103—113
From the Brain Research Laboratory E Blocket the Uni ersity Hospital of Lund Sweden

Metabolic State and Blood Flow in Rat Cerebral Cortex, Cerebellum and Brainstem in Hypoxic Hypoxia

By

V MACMILLAN[1] L G SALFORD and B K SIESJO

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Abstract

MACMILLAN V L G SALFORD and B K SIESJO *Metabolic state and blood flow in rat cerebral cortex cerebellum and brainstem in hypoxic hypoxia* Acta physiol scand *92* 103—113

In order to study the effect of hypoxic hypoxia upon regional cerebral metabol sm and blood flow the arterial Po of lightly anaesthetized rats was lowered to about 35 29 and 23 mm Hg respectively with subsequent measurements of labil metabolites and of blood flow in cerebral cortex cerebellum and brain stem The metabolites which included organic phosphates carbohydrate substrates and amino acids were measured w th enzymatic fluorom tric techniques after freez ng the t ssue *in s tu* and the regional blood flow was estimated from the t ssue uptake of C-ethanol In all 3 regions marked hypoxia (Po 23) gave rise to small increases in the ADP concentrat on but since neither ATP nor AMP was changed from the normal it is con luded that the ene gy state rema ned essen tially unaltered The increase in lactate concentrat on was similar in all three regions and the substrate patterns were essent ally the same Since the regional blood flow also ncreased to a s milar extent in the three regions it is concluded that bra n regions show sim lar metabol c and circulatory changes in hypoxic hypoxia The results thus g e no support to the iew that d fferent bra n areas show selective vulnerability to hypoxia at least not in forms of hypoxia that are uncompl cated by absolute or relat e ischemia

Brain regions are thought to differ in their sensitivity to oxygen lack (selective vulnerability see Schade and McMenemey 1963) and it has been suggested that the sensitivity decreases in the direction from the more rostral to the more caudal portions (Himwich 1951) In recent years techniques have been developed for quick freezing of the brain *in situ* and for measuring labile phosphates carbohydrate substrates and amino acids in various parts of the brain With the help of such tech niques it has been possible to demonstrate that although a lowering of the arterial P_{O_2} to below about 50 mm Hg is accompanied by an increase in the lactate content of the whole brain or of the cerebral cortex a further reduction of the Po to 20—25 mm Hg can be tolerated without causing changes in the contents of ATP ADP or AMP (Gurdjian *et al* 1944 1949 Schmahl *et al* 1966 Siesjo and Nilsson 1971 Duffy *et al* 1972 MacMillan and Siesjo 1972) In one recent study (Duffy *et al*

[1] Present add ss Di on of Neurology Toronto General Hospital Toronto Canada

1972) the authors studied the influence of hypoxic hypoxia upon phosphocreatine (PCr) and ATP concentrations in cerebral cortex cerebellum and brain stem regions Their results did not support the classically held view that brain regions differ in their sensitivity to hypoxia

It has recently been suggested that the resistance of brain tissues to hypoxic hypoxia is partly due to a decreased oxygen consumption secondary to a reduced demand for energy (Duffy *et al* 1972) However, since the oxygen consumption of the rat brain remains unaltered at arterial P_{O_2} values of 23–25 mm Hg (Johannsson and Siesjo 1974) it appears that the main mechanism protecting the tissue in hypoxia is the increase in cerebral blood flow (*cf* Lewis *et al* 1973) The cause of the increase in cerebral blood flow (CBF) is unknown but it has been suggested that tissue production of lactic acid is responsible (Kogure *et al* 1970)

In the previous regional study (Duffy *et al* 1972) no measurements of ADP or AMP were performed and the freezing technique was not ideally suited to compare the metabolic responses of superficial and deep cerebral structures We have therefore repeated these studies using a freezing technique that seems to avoid post mortem changes even in deeply situated brain structures (see Pontén *et al* 1973b) and included measurements of ADP and AMP In addition the regional CBF (rCBF) was measured in the same brain regions (cerebral cortex cerebellum and brain stem) The objective of the study was to evaluate whether or not brain structures differ in their metabolic and circulatory response to hypoxic hypoxia

Methods

Since the techniques and procedures used in the present study were similar to those reported in previous communications from the laboratory (Siesjo *et al* 1972 Folbergrová *et al* 197[illegible] a and b MacMillan and Siesjo 1972 Eklof *et al* 1974) only the main outlines are given here The experiments were performed on male Wistar rats (300–400 g) that had free access to rat pellets and water Anesthesia was induced with divinyl ether and maintained on 70 % N_2O and 30 % O_2 The animals were immobilized and artificially ventilated to give arterial CO_2 tension of 35–40 mm Hg The body temperature was kept close to 37 °C In animals used for studying cerebral metabolites a femoral artery was cannulated for blood pressure recording and for anaerobic sampling of blood When studying rCBF both femoral arteries and veins were cannulated (see below) After allowing a steady state period of 15–20 min the inspired O_2 concentration was reduced so as to give arterial P_{O_2} values of about 35 29 or 23 mm Hg while keeping the nitrous oxide concentration constant These P_{O_2} levels were then maintained constant for 30 min before either metabolites or regional blood flow were measured If the mean arterial blood pressure fell below 1[illegible]0 mm Hg the animal was excluded from the material Blood was sampled at least twice during the hypoxic period for measurements of P_{O_2} P_{CO_2} and pH the last sample taken just before tissue and CSF were collected or blood flow was measured Control groups were obtained by maintaining the O_2 concentration at 30 % throughout the 30 min period

In the metabolic series the atlanto-occipital membrane was exposed for later sampling of cisternal CSF and a skin incision was made over the cranial vault to accommodate a plastic funnel for freezing the tissue in situ (see Pontén *et al* 1973b) At the end of the 30 min experimental period arterial blood was collected in liquid nitrogen for subsequent analyses of glucose lactate and pyruvate CSF was obtained and frozen in liquid nitrogen for similar purposes and the tissue was frozen by pouring liquid nitrogen into the funnel The freezing was continued for 2–3 min with maintained ventilation and the brain was then chiselled out in the frozen state Samples were then dissected at [illegible] °C to represent the cerebral hemisphere (frontal part of the cortex with some underlying white matter) the medial and upper parts of the cerebellum and the brain stem (mid-brain and pons) Since the sample from the cerebral hemisphere contained mainly cortical tissue it will be denoted cerebral cortex The tissue samples were used for subsequent analyses of metabolites (see below)

TABLE I Arterial oxygen and carbon dioxide tensions and pH mean arterial blood pressure (MABP) and body temperature in control and hypoxemic animals Values are means ± S E n = number of expts Physiological parameters obtained at P_O 35 mm Hg (Hypoxia I) is given in the text

Exp group	Pao mm Hg	Paco mm Hg	pH	MABP mm Hg	Temp C
Control (n = 5)	124 ±2	35 4 ±0 2	7 357 ±0 003	137 ±2	36 8 ±0 1
Hypoxia II (n = 5)	28 8 ±1 1	31 1 ±0 7	7 138 ±0 018	130 ±7	37 0 ±0 1
Hypoxia III (n = 5)	22 6 ±0 7	31 8 ±0 9	7 122 ±0 015	136 ±9	36 9 ±0 1

In the series used for blood flow studies no measures were taken to sample CSF or to freeze the tissue *in situ* At the end of the 30 min experimental period 0 5 ml of Ringer s solution containing about 1? / Ci [14]C ethanol was infused continuously into one femoral vein and 40 / l samples were take i from one femoral artery every 3 s Exactly 30 s after the start of the infusion saturated KCl was nfused into the other femoral vein the animal was quickly decapitated and the head was f ozen in l quid nitrogen The brains were subsequently d ssected at −12 C and cerebral cort cal c rebellar and brain stem samples were taken for measurement of the [1] C-acti ity

Arterial P_O P_{CO} and pH were measured with microelectrodes at 37 C and he noglob n was analysed photometrically after conversion to cyanmethhemoglobin Blood and CSF samples were analysed for glucose lactate and pyruvate using enzymatic techniques (see below) The tissue sam ples (as well as blood and CSF) were extracted at −22 C with HCl methanol and the tissue was subsequently analysed for glucose glucose 6 phosphate (G-6-P) pyruvate lactate citrate *a* keto glutarate malate glutamate aspartate phosphocreatine (PCr) creatine ATP ADP and AMP us ng the fluorometric enzymatic methods of Lowry and Passonneau (1972) as described in det il in previous communications (Folbergrova *et al* 1972 a and b) All metabol tes were measur d in the control group and in the groups exposed to a lowering in the P_O to 29 and 23 mm Hg respectively In the P_O 35 group only the lactate concentration will be reported

The act vity of C-ethanol in blood and tissue was measured with a liquid scintillation techni jue nd the rCBF was e timated as described in a recent communication (Eklof *et al* 1974) using the mathemat cal methods of Re vich *et al* (1969) Since the catheter used for sampl ng of arter l blood was only 1−2 cm long no correction was made for catheter mearing

Statistical differences were evaluated with Student s t test

Results

Regional metabolite patterns The physiological parameters of the animals in the metabolite series are shown in Table I As remarked no animal with a mean arterial blood pressure of less than 120 mm Hg was included The hemoglobin concentration was in excess of 13 g (100 ml)$^{-1}$ in each animal (not shown) The hypoxic animals had a slight fall in arterial CO_2 tension and developed a non respiratory acidosis The blood lactate concentrations at 30 min were 4 3±0 2 11 5±1 7 and 11 0±1 4 mmoles kg^{-1} in the control and hypoxic groups respectively (means±S E) The corresponding pyruvate concentrations were 0 22±0 03 0 21±0 02 and 0 21±0 01 mmol kg^{-1} respectively In the CSF the lactate con centrations were 3 2±0 1 8 5±1 4 and 11 5±1 3 and the pyruvate concentrations were 0 18±0 01 0 24±0 01 and 0 30±0 01 mmol kg^{-1} respectively Thus the hypoxic situations were accompanied by a marked lactic acidosis in blood and CSF

TABLE II Brain tissue concentrations of phosphocreatine creatine ATP ADP and AMP as well as *calculated* sums of phosphocreatine and creatine (TCr) and of ATP ADP and AMP (ΣAd) together with energy charge potentials (ECP) in control and hypoxemic rats Values are means ± S E (mmol kg⁻¹ of wet tissue) n = number of expts. Statistical differences from the appropriate controls are given as p < 0 05 = * p < < 0 01 = ** and p < 0 001 = *** The ECP value was calculated as $\frac{ATP + 0.5\ ADP}{ATP + ADP + AMP}$ (Atkinson 1968)

Group	PCr	Cr	TCr	ATP	ADP		AMP	ΣAd	ECP
Cerebrum									
Control	4 96	5 95	10 91	3 13	0 231		0 037	3 40	0 955
(n = 5	±0 06	±0 06	±0 11	±0 01	±0 003		±0 002	±0 01	±0 001
P_{O_2} 29	3 97	6 70	10 67	3 10	0 251		0 037	3 39	0 95?
(n = 5	±0 0 ***	±0 10*	±0 07	±0 03	±0 004**		±0 002	±0 03	±0 001*
P_O 23	3 48	7 69	11 17	3 09	0 271		0 039	3 40	0 949
(n = 5	±0 23***	±0 26***	±0 06	±0 02	±0 006	*	±0 003	±0 07	±0 00
Cerebellum									
Control	6 30	8 11	14 40	2 88	0 258		0 045	3 18	0 946
(n = 4	±0 07	±0 13	±0 16	±0 04	±0 009		±0 001	±0 03	±0 001
P_{O_2} 29	5 82	8 81	14 64	2 87	0 285		0 047	3 21	0 941
(n = 5	±0 23	±0 09**	±0 27	±0 03	±0 010		±0 005	±0 02	±0 003
P_O 23	4 81	10 12	14 94	2 86	0 295		0 049	3 21	0 939
(n = 5	±0 39*	±0 56*	±0 21	±0 07	±0 006	*	±0 004	±0 07	±0 007
Brain stem									
Control	4 51	6 13	10 64	2 70	0 234		0 042	2 93	0 94
(n = 5	0 17	±0 19	±0 13	±0 03	±0 006		±0 005	0 04	±0 007
P_O 29	3 84	6 4	10 57	2 68	0 256		0 042	2 98	0 943
n = 4	0 15 *	±0 08*	±0 18	±0 04	±0 011		±0 004	0 04	±0 001
P_O 23	3 98	7 86	10 87	2 71	0 281		0 050	3 04	0 937
n	0 40	0 75*	±0 29	±0 04	±0 009	*	±0 00	0 05	±0 007

Only 3 samples were obtained for analyses

with pyruvate increasing in CSF but not in blood (*cf* Siesjo and Nilsson 1971 MacMillan and Siesjo 1972 In the additional hypoxic group of six animals (Hypoxia I) the Pa_{O_2} was 35 1 ± 0 3 mean ± S E and the Pa_{CO_2} was 32 5 ± 0 6 mm Hg I these animals there was no fall in arterial pH

Table II illustrates the concentrations of PCr creatine ATP ADP and AMP in the cerebral hemisphere the cerebellum and the brain stem In the control situation there were regional differences in the concentrations of the phosphates but also in the pool sizes sum of phosphocreatine and creatine sum of adenine nucleotides In the brain stem the PCr concentration was slightly lower than in the cerebrum (*p* < 0 05 but the sum of PCr and creatine did not differ significantly (*p* > 0 05 The high PCr concentration of the cerebellum confirms previous reports (Cattel *et al* 1966 Duffy *et al* 1972 but since also the creatine concentration was higher than in the cerebral cortex there was only a small difference in PCr creatine ratio

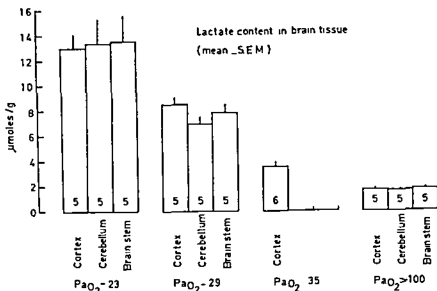


Fig 1 Influence of hypoxia on lactate contents (mmol kg^{-1}) in cerebral cortex cerebellum and bra n stem The lactate values are ind cated by staples and the standard error by vertical bars Number of experiments are g ven at the bottom of the bars In all cases the lactate contents obtained in hypoxia are significantly different from the controls at the 0 1 level ( )

($p < 0\ 01$) The ATP concentration was higher in the cerebral cortex than in the cerebellum and in the brain stem ($p < 0\ 001$) Due to these differences and to a significantly higher ADP value in the cerebellum than in the cerebral cortex ($p <$ $< 0\ 05$) the adenylate energy charge was lower in the cerebellum and the brain stem than in the cortex ($p < 0\ 001$) However the differences in ATP concentration between the regions were largely due to differences in the size of the adenine nucleotide pools and the small differences in energy charge do not indicate that appreciable autolytic changes had occurred in the deeply situated regions (*cf* Ponten *et al* 1973b)

In confirmation of previous work the present data demonstrate that hypoxia does not alter the concentrations of ATP or AMP in any of the regions studied (see Introduction) Moreover hypoxia gave rise to the expected decreases in the PCr and increases in the creatine concentrations in all 3 regions but the sums of PCr and creatine remained the same At P_O 29 there was a highly significant increase in ADP in the cerebral hemisphere and at P_O 23 increases were seen also in the cerebellum and the brain stem Due to this small increase in ADP there were significant decreases in the adenylate energy charge and in the ATP/ADP ratio (not shown) in all three regions at P_{O_2} 23 The data suggest that small decreases had occurred also at P_{O_2} 29 but the changes failed to reach statistical significance in the cerebellum and brain stem possibly due to the small number of samples obtained The results indicate that small changes in energy state occurred in all three regions and that these changes were proportional to the degree of hypoxia (see Discussion)

immersion of the whole animal in a suitable coolant (see *e g* Duffy *et al* 1972) This method has the advantage that non anesthetized animals can be used, allowing a correlation between functional behaviour and metabolic state However, there is the disadvantage that autolytic changes occur before the tissue has been frozen (Pontén *et al* 1973 a and b) and since the autolysis is directly related to the delay in freezing it is difficult to study accurately the metabolic state of structures that are remote from the surface In anaesthetized and paralyzed animals even deep brain structures can be frozen in such a way that autolytic changes are avoided (Pontén *et al* 1973 b) The method has the disadvantage that the procedures lead to hyperglycemia and it carries the risk that anesthesia may blunt the response to hypoxia However anesthesia with 70 % N_2O appears to have minimal effects on cerebral metabolic rate (Theye and Michenfelder 1968 Smith and Wollman 1972) and previous results on unanesthetized animals do not indicate that glucose transport between blood and tissue becomes limiting in hypoxia (Duffy *et al* 1972)

The blood flow method used in the present study is based on the tissue uptake principle described by Kety (1950) and on the method worked out by Landau *et al* 1955 for the radioactive gas trifluoroiodo-methane and later modified for ^{14}C antipyrine by Reivich *et al* (1969) The method which is based on measurements of the rate of uptake of the radioactive marker in the tissue requires that the region under study is homogenously perfused and that there is instantaneous diffusion of the tracer between blood and tissue We recently found that the ^{14}C-antipyrine method grossly underestimates regional blood flow in situations with high flow (hypercapnia and since also other and more diffusable substances ($^{3}H_2O$ ^{133}Xenon and ^{14}C-ethanol failed to give the expected increase in flow we concluded that the assumptions of the method are not valid for any tracer at high flows (Eklöf *et al* 1974 However if ^{14}C-ethanol is used with a 30 s infusion period the rCBF values obtained for cortical tissue come close to those obtained with a modification of the Kety and Schmidt 1948 method (Norberg and Siesjo 1974 at degrees of hypoxia comparable to these employed presently see Johannsson and Siesjo 1973) Furthermore although the regions sampled presently cannot be assumed to have a homogenous blood flow the mean flow obtained should not differ much from the true mean blood flow and it seems excluded that the method could overestimate the flow see Eklöf *et al* 1974 We thus tentatively conclude that the present estimates of rCBF represent close approximations to the true flow values

The present results give no support to the view that gross brain regions show selective vulnerability to pure hypoxic hypoxia *i.e.* a form of cerebral hypoxia which is not complicated by a decrease in cerebral perfusion pressure Thus although there were minor differences in the metabolic response to hypoxia see Fig 2 the overall pattern was similar in cerebral cortex cerebellum and brain stem There are only minor differences between the present results and these reported by Duffy *et al* 1972 Thus in their experiments the glucose concentration in blood and tissue decreased during hypoxia This difference in response may have been due to the fact that Duffy *et al* 1972 used unanesthetized fasted animals while our results were

obtained on anesthetized animals which had free access to food. Furthermore, Duffy *et al.* (1972) found no decrease in PCr in cerebellum, midbrain, pons or medulla. This may be explained by the fact that their freezing technique allowed some post mortem changes to occur in the normoxic animals. However, in both studies the ATP concentration remained unchanged in hypoxia and the increase in lactate concentration was similar in all regions investigated. These facts, as well as the similarity in blood flow changes between the regions, indicate that the cerebral cortex, the cerebellum and the brain stem react in a relatively stereotyped fashion in hypoxia.

In the present experiments the hypoxic brains were extracted and analysed together with the appropriate controls. This procedure made it possible to reveal a small increase in the ADP concentration in the hypoxic brains and a small but significant fall in the adenylate energy charge. Since a similar increase in ADP is not seen after 2, 5 or 15 min of hypoxia (Bachelard *et al.* 1973) it seems to result from a more prolonged decrease in arterial P_O (*cf.* also similar changes in pronounced hypocapnia as reported by MacMillan and Siesjo 1973). In view of the fact that neither ATP nor AMP were significantly altered it remains a possibility that the increase in ADP concentration is related to other factors than an imbalance between production and utilization of energy. A true increase in ADP at unchanged levels of ATP or AMP could result from an altered equilibrium constant in the adenylate kinase reaction (see Rose 1968). There is also the possibility that the recorded increase in ADP is artefactual since the enzymatic method used is not specific for ADP (see Lowry *et al.* 1964, Lowry and Passonneau 1972). Thus, if compounds such as UDP increase during hypoxia, the ADP concentration measured could be erroneously high. As long as these possibilities have not been excluded it does not seem warranted to equate the small increase in ADP with a true change in energy state.

As stated in the introduction, the maintenance of a near normal energy state in brain tissues in profound hypoxia seems to be due to the increase in CBF. Previous results have shown that the cortical blood flow increases when the arterial P_{O_2} is reduced below about 50 mm Hg, and at very low O_2 tensions the CBF may increase considerably (Courtice 1941, McDowall 1966). In the present experiments a reduction of the arterial P_{O_2} to 22–23 mm Hg was accompanied by a 4- to 6-fold increase in rCBF in most of the regions studied. In the cerebellum the rCBF increased 6 to 7 fold. In general, the results demonstrate that infratentorial and deep cerebral structures showed relative increases in rCBF during hypoxia that were at least as pronounced as these observed in cerebral cortical tissues. The results indicate that the increase in rCBF in hypoxic hypoxia is a generalized phenomenon. The cause of this homeostatic increase in rCBF is unknown. *If* the lactic acidosis is responsible (Lassen 1968, Kogure *et al.* 1970), the present results show that maximal vasodilatation in the brain is not obtained until the tissue content of lactic acid increases to 13–15 mM kg^{-1} or higher. However, it will remain for future experiments to delineate the coupling between metabolism and flow in the hypoxic brain.

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References

ATKINSON D E The energy charge of the adenylate pool as a regulatory parameter Interaction with feedback modifiers *Biochemistry* 1968 *7* 4030—4034

BACHELARD H L LEWIS U PONTÉN and B K SIESJÖ Mechanisms activating glycolysis in the brain in arterial hypoxia *J Neurochem* 1973 *22* 395—401

COURTICE F C The effect of oxygen lack on the ceerbral circulation *J Physiol* (Lond) 1941 *100* 198—211

DUFFY T E S R NELSON and O H LOWRY Cerebral carbohydrate metabolism during acute hypoxia and recovery *J Neurochem* 1972 *19* 959—977

EKLOF B N A LASSEN L NILSSON K NORBERG B K SIESJO and P TORLÖF Regional cerebral blood flow in the rat measured by the tissue sampling technique a critical evaluation using four indicators C^{14} antipyrine C^{14} ethanol H^3 water and Xenon[1] *Acta physiol scand* 1974 *91* 1—10

FOLBERGROVÁ J V MACMILLAN and B K SIESJO The effect of moderate and marked hypercapnia upon the energy state and upon the cytoplasmatic $NADH/NAD^+$ ratio of the rat brain *J Neurochem* 1971 a *19* 2497—2505

FOLBERGROVÁ J V MACMILLAN and B K SIESJO The effect of hypercapnic acidosis upon some glycolytic and Krebs cycle associated intermediates in the rat brain *J Neurochem* 1972 b *19* 1507—2517

GATFIELD P D O H LOWRY D W SCHULTZ and J V PASSONNEAU Regional energy reserves in the mouse brain and changes with ischaemia and anaesthesia *J Neurochem* 1966 *13* 185—195

GURDJIAN E S W E STONE and J E WEBSTER Cerebral metabolism in hypoxia *Arch Neurol Psychiat* 1944 *51* 472—477

GURDJIAN E S J E WEBSTER and W E STONE Cerebral constituents in relation to blood gases *Amer J Physiol* 1949 *156* 149—157

HIMWICH H E *Brain metabolism and cerebral disorders* William Wilkins Baltimore 1951

HOLMIN T and B K SIESJO Influence of hypoxia on cerebral energy state in rats with porta-caval anastomosis *J Neurochem* 1974 *22* 413—417

JOHANNSSON H and B K SIESJO Blood flow and oxygen consumption of the rat brain in profound hypoxia *Acta physiol scand* 1974 *90* 281—287

KETY S S Circulation and metabolism of the human brain in health and disease *Amer J Med* 1950 *3* 205—217

KOGURE K P SCHEINBERG O M REINMUTH M FUJISHIMA and R BUSTO Mechanisms of cerebral vasodilatation in hypoxia *J appl Physiol* 1970 *29* 223—229

LANDAU W M W H FREYGANG L P ROSLAND L SOKOLOFF and S S KETY The local circulation of the living brain values in the unanesthetized and anesthetized cat *Trans Amer neurol Ass* 1955 *80* 125—129

LASSEN N A The main factors controlling cerebral blood flow *Scand J clin Lab Invest* 1968 *22* 247—251

LEWIS L D U PONTÉN and B K SIESJO Homoestatic regulation of brain energy metabolism in hypoxia *Acta physiol scand* 1973 *88* 284—286

LOWRY O H and J V PASSONNEAU The relationships between substrates and enzymes of glycolysis in brain *J biol Chem* 1964 *239* 31—41

LOWRY O H and J V PASSONNEAU *A flexible system of enzymatic analyses* Academic Press New York and London 1972

MACMILLAN V and B K SIESJÖ Brain energy metabolism in hypoxemia *Scand J clin Lab Invest* 1972 *30* 127—136

MACMILLAN V and B K SIESJÖ The influence of hypocapnia upon intracellular pH and upon some carbohydrate substrates amino acids and organic phosphates in the brain *J Neurochem* 1973 *21* 1283—1299

MCDOWALL D G in *A Symposium on Oxygen Measurements in Blood and Tissue and their Significance* Ed J P Payne and D W Hill J & A Churchill Ltd London 1966 pp 205—219

NORBERG K and B K SIESJÖ Quantitative measurements of blood flow and oxygen consumption in the rat brain *Acta physiol scand* 1974 *91* 154—164

PONTÉN U R A RATCHESON and B K SIESJÖ Metabolic changes in the brains of mice frozen in liquid nitrogen *J Neurochem* 1973 a *21* 1121—1126

PONTÉN U R A RATCHESON L G SALFORD and B K SIESJÖ Optimal freezing conditions for cerebral metabolites in rats *J Neurochem* 1973 b *21* 1127—1138

REIVICH M J JEHLE L SOKOLOFF and S S KETY Measurements of regional cerebral blood flow with antipyrine C in awake cats *J appl Physiol* 1969 *27* 296—300

ROSE J A The state of magnesium in cells as estimated from the adenylate kinase equilibrium *Proc nat Acad Sci* (Wash) 1968 *83* 235—248

SCHADÉ J P and W H McMENEMEY (Ed) *Selective vulnerability of the brain in hypoxaemia* Blackwell Scientific Publ Oxford 1963

SCHMAL, F W E BETZ E DETTINGER and H J HOHORST Energiestoffwechsel der Grosshirnrinde und Electroencephalogramm bei Sauerstoffmangel *Pflugers Arch ges Physiol* 1966 *292* 46–59

SIESJÖ B K, J FOLBERGROVÁ and V MACMILLAN The effect of hypercapnia upon intracellular pH in the brain, evaluated with the CO method and from the creatine phosphokinase equilibrium *J Neurochem* 1972 *19* 2483–2495

SIESJO B K and L NILSSON The influence of arterial hypoxemia upon labile phosphates and upon extracellular and intracellular lactate and pyruvate concentrations in the rat brain *Scand J clin Lab Invest* 1971 *27* 83–96

SMITH A L and H WOLLMAN Cerebral blood flow and metabolism effects of anesthetic drugs and techniques *Anesthesiology* 1972 *36* 378–400

THEYE R A and J D MICHENFELDER The effect of nitrous oxide on canine cerebral metabolism *Anesthesiology* 1968 *29* 1119–1124

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From the Department of Physiology University of Göteborg Sweden

Tactile Placing Reactions in Chronic Spinal Kittens

By

H FORSSBERG, S GRILLNER and A SJÖSTRÖM

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Abstract

FORSSBERG H S GRILLNER and A SJÖSTRÖM *Tactile placing reactions in chronic spinal kittens* Acta physiol scand 1974 *92* 114—120

Transection of the spinal cord at the level of Th 12 to L 1 was performed in 6—14 days old kittens The kittens were kept for several months after the operation During this period tactile stimuli applied to the dorsum of the hindpaw could evoke a flexion followed by an extension and a placing of the limb in a more rostral position These reactions have been studied by recording the EMG activity and filming the movement It is concluded that it must be regarded as tactile placing which contrary to what has hitherto been assumed is not a reflex relayed in higher nervous structures but rather a complex form of spinal reflex

Rademaker (1931) and Bard (1933) described several reflexes in the intact cat which were called placing reactions These were found to occur only when the sensorimotor cortex was intact It has therefore been generally assumed that they were mediated via the cortex and thus were cortical reflexes or at least representing higher nervous functions (Bard 1938 and Amassian *et al* 1972) The possibility that subcortical centers are also of importance has been advocated by several authors (Bogen and Campbell 1962 Meyer *et al* 1963 and Amassian and Ross *1973)* The latter group reported that decorticated kittens in fact show one of these reactions called tactile placing This reflex is elicited by light touch to the dorsum *of the foot It results in a flexion during which the limb is brought* forward followed by an active extension and a subsequent placing of the foot in a more rostral position in contact with ground The latency from contact to the onset of flexion is for the fore limb 20—30 ms (Amassian *et al* 1972) and for the hindlimb 25—30 ms (Lundberg and Norrsell unpubl as cited by Lundberg 1973) This low value lead the latter investigators to question if the onset of tactile placing could be relayed in the cortex

Bard's (1933) findings have been taken to indicate that the placing reaction is a cortical reflex—but there is another alternative envisaged by McCulloch as cited by Bard (1938) The reflex might have its neuronal machinery (*i e* the interneurones responsible for the flexion extension movement initiated by the tactile stimulus) in any subcortical structure but this reflex center could be under cortical control and require *e g* a facilitatory influence to be operative

In the present experiments chronic spinal kittens operated a week or two after birth were investigated It will be shown that tactile placing occurs in these kittens and hence that it represents a complex type of spinal reflex These results were mentioned briefly by Grillner (1973)

Methods

10 kittens 6—14 days old premedicated with Chlorpromazin (2 5 mg/kg) and Petidin chloride (1? 5 mg/kg) were operated under ether administered with a mask After the operation 5 ml Glucose Ringer was given intraperitoneally The kittens woke up approximately half an hour after the operation and could be brought back to their mothers within a few hours Antibiotics were prophylactically given for one week The operated kittens increased in weight in parallel with the unoperated ones in the same litter They were helped with bowel and bladder emptying twice a day and their hindlimbs were manipulated or trained at least one hour every day particularly by walking on a treadmill

The spinal cord was exposed by a laminectomy of one vertebra, and half a segment of the spinal cord was removed at the level of Th 12—L 1 under microscopic inspection After the animals were sacrificed it was checked whether the rostral and the caudal part of the cord was far apart. If not a histological examination was used to confirm the transection

The hindpaw tactile placing responses were studied following the procedure outlined by Bard (1933) and used also by later investigators (Amassian *et al* 1972) To record contact and placing we also used a set of two plates mounted on strain gauges which were placed at 90 The vertical plate sensitive enough to detect a force down to 20 mg or even the contact of the hair on the paw was used to record the moment of contact The horizontal plate recorded the actual placing of the foot

The animal was held in the air in such a position that the legs were freely hanging and passively extended or that the hip joint angle was approximately 90 to 100 The animal was then brought close to the vertical plate so that the dorsum of the paws touched the vertical plate eliciting a placing reaction These reactions could also be elicited by touching the hair on the dorsum of the paw with a fine brush

EMGs were recorded with a pair of copper wires (100 μm in diameter) insulated except for 2—3 mm at the tip They were inserted by hypodermic needles (later withdrawn) into the muscles investigated (tibialis anterior TA and gastrocnemius G) The mechanical and electromyographical signals were recorded on an 8-channel Mingograph with a straight frequency response up to 1200 Hz (specially selected galvanometers)

The placing reactions were also filmed with 16 mm Ilford Pan F negative film at 80 frames/s From these films the time course of the movement and the joint angles could be evaluated

Results

Already 1—2 days after the operation the hindlimbs had some tonus and showed alternating or simultaneous activity as during locomotion when the cats were held over a treadmill with the limbs touching the moving band This was in a stage when the eyes were still closed and neither the fore nor hindlimb had force enough to support the animal They could also occasionally show placing reactions which progressively got more constant during the following days Fig 1 shows a kitten of 20 days of age It was held with the hindlimbs free and passively extended and then the kitten was brought forwards until the dorsum of the paw merely touched the table edge (Fig 1 A) The limb was then withdrawn with flexion in knee and ankle until it cleared the top (Fig 1 B) and thereafter placed in a more rostral position with extension in knee ankle and foot joints (Fig 1 C) Fig 1 D shows the changes in joint angles in knee and ankle during a placing reaction as obtained from cinematographical record

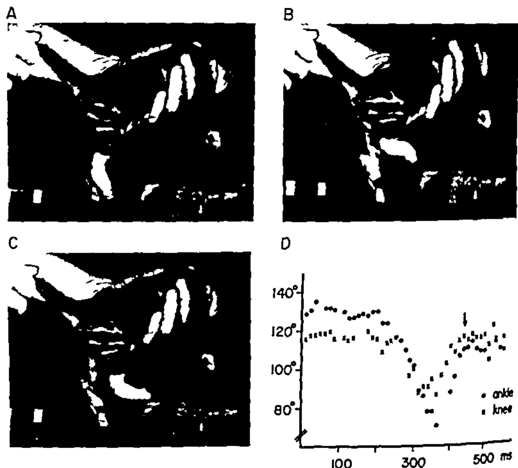


Fig 1 *Placing reactions in a chronic spinal kitten* A—C are photographs from three different placing reactions in a 20 days old kitten (spinal transection at the age of 6 days) The placing reactions have also been followed with 80 frames/s on 16 mm films but the single frames were not suitable for photographic reproduction and hence ordinary photos from successive placing reactions have been taken with an ordinary 24×36 mm camera and used for illustrations D shows the time course of the change in the joint angles of knee and ankle during a placing reaction It was from a 16 mm film (20 frames/s) taken under identical condition to A—C The placing of the foot is indicated by an arrow to the right while the contact was not clearly determined

The EMG activity in an ankle flexor (TA) and extensor (G) was recorded during different placing reactions in Fig 2 The upper traces show the contact with a vertical plate (see Methods) and with a horizontal surface as the foot is placed the lower traces show the EMG activity Fig 2 A shows that the activity in TA starts just after contact and 390 ms later this activity switches to a marked extensor activity followed by the placing of the foot The extensor remains active for a long period Fig 2 B shows that the lag between contact and placing can be much longer and indeed it was found to vary between 2[illegible]0—2000 ms although it usually ranged

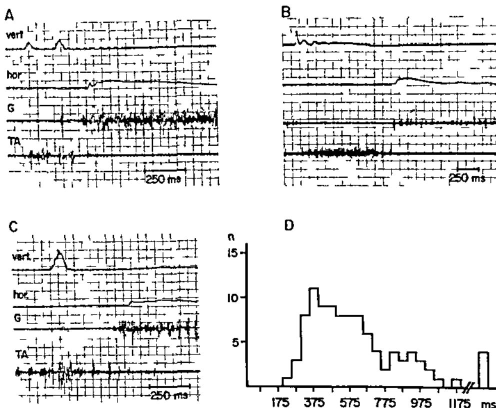


Fig 2 *The electromyographical activity during placing reactions* The lower traces in A to C are recorded in the ankle extensor gastrocnemius (G) and the flexor tibialis anterior (TA) The upper traces show the moment of contact of the dorsum of the paw while the 2nd shows the placing of the foot (see Methods) Note the rapid onset after contact in A and B and the enhanced flexor activity in C After the flexion is completed an extensor activity starts D is a histogram showing the lag between contact and placing in 99 trials during one experimental session The four values indicated to the right in the histogram were exceptionally long varying from 1200—2900 ms (*cf* B) Note that the time calibrations are different in B and A C

between 350—700 ms (Fig 2 D) As the foot ascends after making contact it will often touch the vertical surface again If so the flexor activity is enhanced and only when the foot has cleared the edge of the table the extensor activity can take over and place the foot This transition from flexor to extensor activity is rather variable and sometimes the foot is kept in flexion for quite some time before suddenly the flexor activity ceases and the extensor activity is switched on With the hips extended and the limbs free in the air the chronic spinal cat often shows alternating contractions in flexors and extensors which can be feeble or strong If the foot during such feeble activity gets in contact (Fig 2 C) with the vertical surface during flexor activity it is promptly withdrawn and the EMG activity increases markedly

and the placing reaction has a low threshold Correspondingly if during the placing there is an ongoing extensor activity this often ceases and instead a flexion is initiated The vertical plate was sensitive enough to record a displacement when touched exclusively by the hair on the foot To further assure that tactile stimuli was sufficient we used al o a fine brush just touching the hairs on the dorsum of the foot Such a stimulus could as well result in placing reactions

Amassian et al (1972) has also described medial and lateral placing in the intact cat Also in the e chronic spinal kittens contact to the lateral or medial aspect of the paw could lead to a flexion of the hindlimb and its subsequent placing more laterally or medially respectively These reactions were however, much more difficult to reveal and more variable than those elicited from the dorsum of the paw

These placing reactions could be evoked in all the kittens studied but they appeared less regularly if the cats had been inactive for a longer period of time Conversely if the cats (hindquarters) had just been trained to walk on the treadmill or manipulated the placing would be more easily elicited (see also discussion below) The easiness with which the placing reactions could be demonstrated was seen al o to vary in time after the spinalization As already noted they could often be observed as early as one or two days after the transection of the cord but be more easily elicited some weeks later As the animals grew older it would again become more difficult to evaluate the placing reaction because when the hindlimbs were held in extended position the alternating activity (spinal stepping) became more marked and it only stopped when the pads were put on the ground When the dorsum of the paw was touched during these periods of alternating movements it often happened during the flexion which usually elicited an increased flexor activity and a subsequent placing

It is interesting to note that placing could be elicited already one or two days after the spinalization consisting of a removal of half a segment After this short period of time significant plastic changes cannot be expected Hence it appears that the tactile placing circuits are operative in the spinal state in these kittens After some weeks when plastic changes have occurred these reactions can on the other hand be more regularly demonstrated

Discussion

Bard (1933) describes tactile placing in the following way If a cat is held in the air with the legs free and dependent and with the head held up (so that it cannot see its forefeet or any object below and in front) the slightest contact of the backs of either pair of feet with the edge of a table results in an immediate and accurate placing of the feet soles down on the table close to its edge The tactile placing studied in the present experiments and by many other investigators (Bogen and Campbell 1962 Meyer et al 1963 and Amassian et al 1972) have been elicited in the same way In addition we have also elicited placing reactions with a fine brush only influencing some hairs on the dorsum of the paw Bard (1938) has in

addition described a proprioceptive placing reaction that can be elicited after tactile placing has been differentially abolished After the foot has made contact the body is brought further forward and when the forward displacement of the body has induced a considerable degree of retroflexion at shoulder or hip the hand or foot is lifted carried forward and placed far inside the table edge This reaction appears to be identical to tactile placing except for an activation of different receptors It would appear likely that the neuronal apparatus generating the flexion extension movement in this placing would be the same as for tactile placing There can be no doubt however that the spinal placing reactions studied in these experiments must be regarded as tactile placing as defined by Bard (1933 1938) This is further supported by the fact that the latency from contact to onset of flexor activity (Fig 2) is similar to the values given by Lundberg and Norrsell (as cited by Lundberg 1973) and that the lag between contact and placing is in the same order as observed by Amassian *et al* (1972) for the intact cat In this context it is also interesting that tactile placing reactions in the medial and lateral directions can be seen with stimuli to the medial or the lateral aspect of the paw as for intact cats (Amassian *et al* 1972)

Bard (1938) accepted that the proprioceptive placing reactions could be elicited via subcortical centers but insisted on that tactile placing was exerted via a loop in the somatosensory and motor cortex since removal of either of these areas would remove the placing reaction However tactile placing were shown to occur also under other experimental conditions after removal of these cortical areas by *e g* Bogen and Campbell (1962) Meyer *et al* (1963) and Amassian and Ross (1973) Amassian *et al* (1972) have also shown that the placing reaction is indeed accompanied by neuronal activity in VPL of thalamus and several other subcortical structures The present experiments show however that the entire neuronal apparatus necessary for tactile placing is contained within the spinal cord although it cannot be demonstrated in the ordinary spinal state but only after the plastic changes following a spinal transection

What is then the explanation for the findings of Rademaker (1931) Bard (1933) and others? Their results are compatible with ours if one considers that under normal conditions some elements of the spinal circuitry of tactile placing is under the control of supraspinal structures such as the motor cortex which is known to exert a control over the brainstem and the spinal cord These descending controls could facilitate or block the occurrence of tactile placing (or both) when they are operative An interpretation of Bard s finding that a removal of the somatosensory cortex can abolish tactile placing could be that due to the tight connexions between somatosensory and motor cortex (Mountcastle and Darian Smith 1966) the removal of the former would be expected to result in a disturbance of the activity in the motor cortex All data available can be interpreted in this way *i e* the tactile placing reaction of the hindlimb is dependent on a true spinal circuitry which normally is under supraspinal control as so many other reflex pathways (*cf* Lundberg 1966) That the tactile stimulus as well as its motor response is reflected by

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From the Institute of Physiology University of Bergen Norway

Intrarenal Filtrate Distribution, during Saline Infusion in Rats with Unilateral Ureteral Ligation

By

G Clausen

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Abstract

Clausen G *Intrarenal filtrate distribution during saline infusion in rats with unilateral ureteral ligation* Acta physiol scand 1974 *92* 121—129

Six days after unilateral ureteral ligation (UL) the contralateral kidney had increased GFR by 66 % in conscious rats The average relative single nephron filtration rate (gfr) of superficial nephrons as measured by the ^{14}C ferrocyanide single injection technique was 75 % of deep nephron gfr in UL rats compared to 90 % in intact rats Thus hyperfiltration was more pronounced in deep nephrons Isotonic saline infused i v (15 % BW in 60 min) in a second group of UL rats increased GFR by average 36 % compared to preinfusion GFR Higher single nephron GFR and lower fractional sodium reabsorption enabled the UL rats to excrete sodium and water at practically the same absolute rates as compared to intact rats previously studied during identical saline infusion experiments Saline infusion increased superficial gfr in UL rats from 75 % to 96 % of deep gfr Provided superficial nephrons constitute 70 % of total nephron number these results imply that filtration rate rose by 48 % in superficial and by 16 % in deep nephrons It is suggested that maximally one third of total sodium excretion in the saline infused UL rats might be due to superficial nephrons not being able to reabsorb their disproportionately increased filtered load

The present study was made to investigate whether increased single kidney GFR produced by contralateral ureteral ligation (UL) and by i v saline loading after UL is associated with proportional increases in filtration rate in deep and superficial nephrons Several reports show that i v infusion of isotonic saline in anesthetized rats results in a disproportionate increase in the filtration rate of superficial glomeruli (Baines and Davis 1970 Coelho *et al* 1970 Jamison and Lacy 1971 Davidman *et al* 1971) Other investigators using similar saline loading procedures have shown unaltered filtrate distribution (Bartoli and Early 1971 Andreucci *et al* 1971 Daugharty *et al* 1972) Thus disagreement persists as to whether disproportionate increase of filtration in superficial nephrons participates in producing the natriuresis associated with saline loading In a recent study we failed to demonstrate such redistribution of filtrate in conscious rats (Clausen and Tyssebotn 1973) Contrary to most previous investigators we did not observe any increase of total GFR during the saline infusion Similar results have recently been reported by Carriere *et al* (1972) in saline loaded dogs Thus it seems possible that the ratio of superficial to

TABLE I Renal function in rats six days after unilateral ureteral ligation (UL) and the effect of acute i v isotonic saline infusion (15 % of BW in 60 min) after UL

Weight g	U osm mosm/l	Urine flow µl/min 100 g	C_{In} ml/min 100 g BW	C_I increase	Na excretion µeq/min 100 g BW	Na excretion % of filtr	Volume exp % of BW	[gfr]±SD of gfr$_D$	n
UL controls									
233	1116	3	0 73	—	0 2	—	—	60±22	50
251	696	4	0 64	—	0 2	—	—	77±26	52
270	862	8	0 78	—	0 1	—	—	87±29	52
242	498	13	0 75	—	0 2	—	—	75±28	52
mean±SD 247	793	7±4 5	0 73±0 05	—	0 2±0 05	< 0 2	—	75±26	52
UL saline infused									
226	295	152	0 96	46	18 1	13	3 0	130±40	70
227	317	165	0 85	28	19 9	17	6 5	95±28	45
254	350	171	1 10	17	23 8	15	5 0	99±18	43
246	292	172	0 75	65	17 8	17	6 5	97±40	40
248	290	193	1 12	19	22 6	13	6 5	60±42	60
mean±S D 240	309±25	171±15	0 95±0 16	36±20	20 4±2 7	15±2	5 5±1 5	96±34	50

gfr$_S$ and gfr$_D$ relative single glomerular filtration rate in superficial and deep nephrons respectively
n numbers of nephrons of each type
preinfusion C_I averaged 0 70 ml/min 100 g BW

Results

Compensatory hyperfiltration The UL rats maintained normal body weight hematocrit and plasma sodium concentration Mean arterial blood pressure averaged 132 (120—136) mm gH both in the UL control rats and in the UL saline loaded rats prior to infusion Pertinent results on renal functions are listed in Table I

Single kidney GFR after UL was 66 % greater than that of intact rats previously studied by Clausen and Tyssebotn (1973) Superficial gfr was 75 % of deep gfr in the UL rats (Table I) significantly lower ($P < 0.01$) than the 90 % observed in the intact rats These results show according to equation 1 that UL for six days had increased the total filtration rate of the deep nephron population by 86 % ($P < 0.01$) and that of the superficial population by 53 % ($P < 0.02$) as compared to intact rats indicating that compensatory hyperfiltration was more pronounced in deep nephrons (Table II) This is made even more clear by calculating average single nephron filtration rate in per cent of average deep nephron filtration rate in intact control rats (Clausen and Tyssebotn 1973) as shown in Fig 1 The calculated increase of filtration rate in the two populations is practically independent of whether deep nephrons constitute 30 % or 40 % of total nephron number

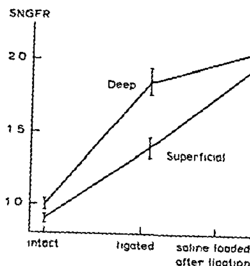


Fig. 1. The effect of unilateral ureteral ligation for six days and of subsequent saline loading on single nephron glomerular filtration rate (SNGFR) ±SE. SNGFR = 1.0 refers to mean deep SNGFR in intact rats (Clausen and Tyssebotn 1973).

Saline infusion. Mean arterial blood pressure increased during saline loading in 3 of the 5 rats by about 15 mm Hg but remained within control level ±5 mm Hg in the other 2. There was no correlation between the degree of effective volume expansion and blood pressure rise. Saline loading increased total GFR in all rats by average 36 % ($P < 0.01$). Superficial gfr was raised from 75 % of deep gfr in UL controls to 96 % ($P < 0.01$) of deep gfr in the saline loaded UL rats (Table I).

The fraction of total GFR derived from deep nephrons did not increase during saline loading (Table III). Since total GFR increased, however, the absolute filtration rates of both nephron populations did increase as demonstrated in Fig. 1. From these results it may be inferred that the deep nephron population contributed 1/6 and the superficial nephrons contributed 5/6 of the increase of GFR that took place. Accordingly the increase of filtrate production within each nephron

TABLE II. Single kidney GFR and its distribution on deep (D) and superficial (S) nephron populations in ureter ligated (UL) and intact rats

	Total GFR	GFR_D		GFR_S	
	ml/min 100 g	ml/min 100 g ±SD	% of GFR	ml/min 100 g ±SD	% of GFR
Intact[1]	0.44	0.14±0.02	32±2	0.30±0.04	68
UL	0.73	0.27±0.03	37±4*	0.46±0.05	63 *
Increase					
ml/min 100 g	0.29*	0.13*		0.16*	
Per cent	66	86		53	

* = $P < 0.05$
** = $0.05 < p < 0.1$
1 = Intact rats studied during identical saline infusion procedure (Clausen and Tyssebotn 1973)

TABLE III Single kidney GFR and its distribution on deep (D) and superficial (S) nephron populations during i.v. saline infusion (15 % BW in 60 min) in ureter ligated rats

	Total GFR	GFR_D		GFR_S	
	ml/min 100 g	ml/min 100 g ±SD	% of GFR	ml/min 100 g ±SD	% of GFR
Preinfusion	0.70	0.26±0.03	37	0.44±0.05	64
Saline infusion	0.95	0.30±0.10	32**	0.65±0.20	69
Increase					
ml/min 100 g	0.25	0.04**		0.21	
Per cent	36	16		48	

* = $p < 0.05$
** = $p > 0.1$

population was 16 % ($P > 0.2$) in the deep and 48 % ($P < 0.02$) in the superficial (Table III). Thus these findings support the hypothesis that increase of total GFR during saline loading is due mainly to increased superficial filtrate production.

The isotonic saline infusion corresponding to 15 % of BW caused an average effective volume expansion of 5.5 % of BW as calculated from the volume of fluid infused (urine produced+blood sampled). A virtually identical degree of volume expansion (5.5 %) was previously observed in the intact saline loaded rats (Clausen and Tyssebotn 1973). During saline infusion the excreted fraction of filtered sodium (fractional sodium excretion) was 15±2 % in UL rats compared to 11±5 % in intact rats ($P < 0.05$). Total clearance of sodium was, however, nearly identical in UL and intact saline loaded rats corresponding to sodium excretion rates of 18 and 20 μeq/min 100 g BW respectively. Osmotic excretion was 53 μOsm/min 100 g BW in both groups. Thus excretion of water, sodium and other solutes as well as volume expansion were the same in UL and intact rats after 40—60 min of saline infusion. Since 75 μOsm/min 100 g BW was infused and 22 μOsm/min 100 g BW was retained, the volume expansion was progressive by about 4 % of BW per hour. These results show that a combined increase of total GFR and of fractional sodium excretion enabled the UL rats to excrete sodium, other solutes and water as efficiently as intact rats.

Discussion

During 6 days with functional exclusion of one kidney by ureteral ligation GFR had increased by about 66 % in the contralateral kidney as compared to single kidney GFR in intact rats. Hence, compensatory hyperfiltration developed somewhat earlier than what might be expected according to Peters (1963) who found that permanent increase of GFR began during the second week and reached 80 % after 3 weeks in UL rats. However, the present results agree well with those reported by Dicker and Shirley (1972) showing that single kidney GFR in rats increased after

seven days with UL to 60—70 % above preoperative levels Weinman *et al* (1973) recently reported that rats with one ureter diverted to the *interperitoneal space for 4* days increased contralateral GFR by about 40 % without altered renal weight. Micropuncture and ferrocyanide determination of filtrate distribution indicated that filtration rate had increased by 78 % in deep 62 % in intermediate and 17 % in superficial nephrons Four days of contralateral ureteral ligation produced about 30 % increase of GFR and about 15 % increase of kidney weight Filtrate distribution in this group was however, not reported The deep nephron population of the present UL-rats correspond roughly to the deep and intermediate groups of Weinmar *et al* Thus the relative increase of filtration was similar in these nephrons in both studies That superficial nephrons increased filtration relatively more in the present UL rats (53 %) might be due to increased superficial nephron growth in *accordance with increased renal weight occuring in ligated rats only*

Fractional sodium excretion probably increased during UL since food (salt) intake as reflected by BW was normal and total GFR was lower than in intact controls As far as tubular sodium reabsorption is concerned however the major quantitative change during UL was not the increase of fractional sodium excretion (in the order of 0 1 μeq/min 100 g BW) but the increase of total sodium reabsorption which was about 20 μeq/min 100 g BW The assumption that short looped superficial nephrons have a lower capacity to reabsorb an increased filtered load of sodium seems to be generally accepted (discussed below) That deep nephrons took over a relatively larger fraction of filtrate production after UL may accordingly reflect an adjustment during compensatory hyperfiltration which prevents excessive salt and water waste Such waste could occur if filtered load in the short looped and possibly low capacity superficial nephrons were allowed to increase in proportion to that of deep nephrons *i e* by an additional 30 % in the present rats

The greatly increased single kidney GFR during UL may be attributed to a general afferent arteriolar dilatation and increased blood flow throughout the renal cortex It seems likely that the greatly increased filtration may have utilized the glomerulotubular balance capacity to the limit in low capacity superficial nephrons and that some feed back mechanism possibly involving macula densa therefore restricted further afferent arteriolar dilatation and further increase of filtration in these nephrons This hypothesis is not contradicted by the fact that superficial nephrons must have increased their total sodium reabsorption during saline loading as discussed below Deep nephrons assumed to have a larger capacity to reabsorb increased filtered load may also have a significantly larger resistance to tubular flow (Baines 1973) If so the afferent arteriolar dilatation necessary to produce an equal increase of filtration in the two nephron types would be disproportionately larger in the deep nephrons For this reason and since deep nephrons increased their filtration rate more than did superficial ones during UL the capacity to dilate the *afferent arteriole* would have been utilized relatively more in the deep nephrons prior to saline loading Thus subsequent acute volume expansion by iv saline infusion producing an additional marked increase of filtration rate through afferent

arteriolar dilation would have a greater effect in the superficial nephrons The results obtained in the saline loaded UL rats are consistent with this line of thought Superficial nephrons increased filtration rate by 48 %, deep ones by only 16 % Inherent in this hypothesis lies the concept that although a feed back mechanism restricts arteriolar dilatation in superficial nephrons during compensatory hyperfiltration it does not do so during saline loading This indicates that saline loading and volume expansion produce a stimulus to vasodilatation in addition to that associated with compensatory hyperfiltration that overrules the feed back vasoconstrictory effect Flow through the loop of the tubulus, and thus through the high resistance segment increases during natriuresis This is partly due to increased GFR but mainly to reduced proximal odium reab orption and low medullary osmolarity Consequently free flow intratubular pressure is raised as shown by Andreucci *et al* (1971) in rats subjected to i v saline loading Since this will increase intrarenal interstitial pressure and tend to reduce transmural pressure in afferent arterioles it stimulates further vasodilatation

Whether total GFR is increased or not and whether filtrate is redistributed or disproportionately increased in superficial nephrons may however depend on experimental and preexperimental circumstances some of which are discussed below

The clearance of inulin in rats varies considerably among different laboratories probably due to different strains of rats use of anesthetics surgery and handling of the experimental kidney Comparatively high GFR values were obtained in this laboratory Since the rats were unanesthetized and the kidneys were untouched it seems unlikely that the disproportionate increase of superficial gfr in the UL rats could be due to an experimentally induced arbitrarily low GFR becoming normal due to saline loading Fractional sodium excretion and thereby total sodium excretion was markedly higher in the conscious intact and UL rats than in anesthetized rats receiving similar isotonic saline infusion (Herrera Acosta *et al* 1972 Baines 1973) This discrepancy might be explained in accordance with recent findings by Keck *et al* (1973) showing that anesthetized dogs retained and extravasated a much larger fraction of i v infused isotonic saline than did conscious ones A greater increase of plasma volume was associated with a higher rate of sodium excretion in the conscious dogs

Andreucci *et al* (1971) and Herrera Acosta *et al* (1972) found that filtration rate increased more in superficial than in deep nephrons during progressive but not during limited volume expansion by isotonic saline suggesting that the degree of expansion per se cau es filtrate redistribution In the pre ent UL rats and in the previously investigated intact rats the effective volume expansion was equal showing that the degree of expansion does not necessarily affect filtrate distribution

Whether redistribution of filtrate occurs during saline loading seems to depend on preexperimental distribution which again may depend on previous salt intake (Horster and Thurau 1968 Baines 1973) on age (Baines 1973) and on GFR as suggested by the present results

Previou observations in conscious rats (Peters 1963 Clau en and Tyssebotn

1973) show that natriuresis may occur without increased GFR and also without filtrate redistribution (Clausen and Tyssebotn 1973). Similar results were obtained in dogs by Carriere *et al.* (1972). Since neither increased GFR nor filtrate redistribution are essential for natriuresis one may wonder whether these changes when they occur, play a role in regulating sodium excretion or merely are side effects. The idea that increased superficial nephron filtration might cause natriuresis seems to be derived from Goodyear and Yarger (1965) who suggested that deep nephrons might be characterized by higher sodium retaining capacity. Horster and Thurau (1968) proposed that filtrate redistribution towards superficial nephrons during high dietary sodium intake might reflect a mechanism whereby sodium excretion was adjusted to intake. Although this hypothesis has attracted the attention of many previous authors, direct evidence that fractional sodium reabsorption is reduced more in superficial than in deep nephrons during saline loading seems to be lacking. The only study claimed to provide such evidence was made by Jamison and Lacy (1971). They found unaltered filtration rate and unaltered fractional sodium reabsorption in juxtamedullary nephrons down to the bend of the loop in saline loaded rats. However, this finding alone, or in relation to corresponding results from superficial nephrons (late proximal and early distal puncture) and to total kidney fractional sodium excretion, hardly offers conclusive evidence as to the validity of the hypothesis.

The present results show that the calculated increase of GFR in the superficial nephron population alone corresponds to 22 % of total GFR during saline infusion. Since over all fractional sodium excretion was only 15 %, this means that superficial nephrons must have increased their absolute rate of sodium reabsorption. It further implies, theoretically, that the 15 % fractional sodium excretion could be due solely to superficial nephrons not being able to reabsorb their increased filtered load. On the other hand, saline loaded intact rats excreted sodium at a rate closely similar to that of the saline loaded UL rats but without increased superficial filtration. It seems justified, therefore, to assume that the disproportionate increase of filtration rate in superficial nephrons migh be responsible maximally for the increase of fractional sodium excretion from 11 % in intact saline loaded rats to 15 % in the saline loaded UL rats. If so, its maximal contribution to the natriuresis corresponds to about one third of the total amount of sodium excreted by the UL rats.

References

ANDREUCCI, V. E., J. HERRERA-ACOSTA, F. C. RECTOR JR. and D. W. SELDIN. Effective glomerular filtration pressure and single nephron filtration rate during hydropenia, elevated arterial pressure, and acute volume expansion with isotonic saline. *J. clin. Invest.* 1972. 50. 2230—2234.

BAINES, A. D. Redistribution of nephron function in response to chronic and acute solute loads. *Amer. J. Physiol.* 1973. 224. 237—244.

BAINES, A. D. and C. DAVIS. Pan-cortical shifts in glomerular tubular function: influence of volume expansion. In: *Regulation of body fluid volumes by the kidney.* 1970. Pp. 40—55. Edited by Cort, J. H. Karger, Basel.

BARTOLI E and L E EARLEY The relative contributions of reabsorptive rate and redistributed nephron filtration rate to changes in proximal tubular fractional reabsorption during acute saline infusion and aortic constriction in the rat. *J clin Invest* 1971 *50* 2191—2203
CARRIERE S B BOULET A MATHIEU and M G BRUNETTE Isotonic saline loading and intrarenal distribution of glomerular filtration in dogs *Kidney Int* 1972 *2* 191—196
CLAUSEN G and I TYSSEBOTN Intrarenal distribution of glomerular filtration in conscious rats during isotonic saline infusion *Acta physiol scand* 1973 *89* 289—295
COELHO J B S R STELLA K C H CHIEN and S E BRADLEY Glomerular filtration rate (GFR) in superficial (S) and juxtamedullary (JM) nephrons during salt loading and hemorrhagic hypotension *Clin Res* 1970 *18* 496
DAUGHARTY T M I F UEKI D P NICHOLAS and B M BRENNER Comparative renal effects of isotonic and colloid free volume expansion in the rat *Amer J Physiol* 1972 *222* 225—235
DAVIDMAN M R C LOANE E A ALEXANDER and N G LEVINSKY Proximal function in volume loading in the rat *Clin Res* 1971 *19* 528
DICKER S E and D G SHIRLEY Compensatory hypertrophy of the contralateral kidney after unilateral ureteral ligation *J Physiol* (Lond) 1972 *220* 199—210
FUHR J J KACZMARCZYK and C D KRUTTGEN Eine einfache colorimetrische Metode zur Inulinbestimmung fur Nieren Clearance Untersuchungen bei Stoffwechselgesunden und Diabetikern *Klin Wschr* 1955 *33* 729—730
GOODYEAR, A V N and C A JAEGER Renal response to nonshocking hemorrhage role of the autonomic nervous system and of the renal circulation *Amer J Physiol* 1955 *180* 69—74
HANSSEN O E A histochemical method for evaluation of excreted sodium ferrocyanide in isolated tubules of the mouse kidney *Acta path microbiol scand* 1958 *44* 363—371
HANSSEN O E Method for comparison of glomerular filtration in individual rat nephrons *Proc 2nd int Nephrol Congr* 1963 pp 527—529
HERRERA ACOSTA J V E ANDREUCCI F C RECTOR JR. and D W SELDIN Effect of expansion of extracellular volume on single nephron filtration rates in the rat *Amer J Physiol* 1972 *222* 938—944
HORSTER M and K THURAU Micropuncture studies on the filtration rate of single superficial and juxtamedullary glomeruli in the rat kidney *Pflugers Arch ges Physiol* 1968 *301* 162—181
JAMISON R L and F B LACY Effect of saline infusion on superficial and juxtamedullary nephrons in the rat. *Amer J Physiol* 1971 *221* 690—697
KECK W R JOPPICH W D v RESTORFF U FUESTERER P PRUCKSUNAND H BRECHTELSBAUER and K KRAMER, Sodium excretion in conscious and anesthetized dogs after large saline infusions *Pflugers Arch ges Physiol* 1973 *341* 51—62
PETERS G Compensatory adaptation of renal functions in the unanesthetized rat *Amer J Physiol* 1963 *205* 1042—1048
WEINMAN E J K RENQUIST R STROUP M KASHGARIAN and J P HAYSLETT Increased tubular reabsorption of sodium in compensatory renal growth *Amer J Physiol* 1973 *224* 565—571

Acta physiol scand 1974 92 130—141

From the Brain Research Laboratory E Blocket University Hospital of Lund Sweden

The Influence of Arterial Hypoxia and Unilateral Carotid Artery Occlusion upon Regional Blood Flow and Metabolism in the Rat Brain

By

LEIF G SALFORD and BO K SIESJO

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Abstract

SALFORD L G and B K SIESJO *The influence of arterial hypoxia and unilateral carotid artery occlusion upon regional blood flow and metabolism in the rat brain* Acta physiol scand 1974 92 130—141

The influence of marked arterial hypoxia combined with unilateral carotid artery clamping upon regional cortical metabolites and blood flow was studied in the rat brain When the arterial P_O was reduced to about 20 mm Hg the hemisphere on the clamped side showed a marked lactic acidosis and a fall in phosphocreatine but the adenylate energy charge remained close to normal In this hemisphere the cortical blood flow increased about 4 fold On the clamped side there was an excessive lactic acidosis and a marked fall in energy charge However since the levels of glucose glucose 6 phosphate and pyruvate did not fall below normal and since the regional blood flow was at least doubled it is concluded that the derangement of the energy metabolism was due to cellular hypoxia exaggerated by the relative ischemia induced by the carotid artery clamping The biochemical lesion was most pronounced in the distribution territory of the middle cerebral artery that also showed the largest degree of relative ischemia It is suggested that the pronounced lactic acidosis may contribute to the neuronal damage observed with this model of hypoxia and relative ischemia.

We have recently observed that if profound arterial hypoxia in the rat is combined with unilateral ligation of the common carotid artery the cerebral hemisphere on the ligated side shows a marked lactic acidosis and a deranged energy state (Salford Plum and Siesjo 1973) and the majority of such animals develop cell changes of the ischemic type demonstrating irreversible neuronal damage (Salford Plum and Brierley 1973) However since the affected tissue did not show decreases in substrate levels (glucose and glucose 6 phosphate) we concluded that the changes observed were due to tissue hypoxia with added relative ischemia induced by the ligation of the carotid artery (Salford Plum and Siesjo 1973) Preliminary measurements of the regional blood flow estimated from the rate of uptake of ^{14}C antipyrine supported the conclusion that the clamping procedure did not cause an absolute reduction of the cortical blood flow on the ligated side but merely limited

the increase in blood flow which occurs in hypoxic animals with intact carotid arteries Thus the experiments suggested that hypoxia alone *i e* a reduction in tissue P_O in the absence of a reduction in blood flow to subnormal values can lead to irreversible tissue damage

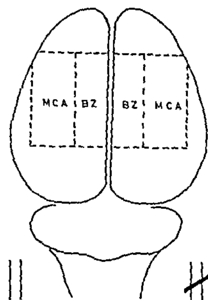
In the present experiments we have correlated the regional metabolic state and the regional blood flow (rCBF) in the cerebral cortex of hypoxic rats that had one carotid artery ligated using a method for rCBF measurement which is less objectionable than that based on ^{14}C antipyrine In addition since the previous histopathologic findings indicate that the cortical cell changes are mainly confined to the distribution territory of the middle cerebral artery (Salford Plum and Brierley 1973) we have compared changes in regional metabolic state and rCBF in this area to those occurring in the boundary zone between the distribution territories of the anterior and middle cerebral arteries This latter region is preferentially affected in pure ischemia (Brierley *et al* 1969) and the comparison should thus provide further information on the characteristics of hypoxic and ischemic lesions respectively

Methods

Since the experimental procedures have been described in detail elsewhere (Salford Plum and Siesjo 1973) only a brief outline is given here The experiments were performed on male Wistar rats (300—400 g) that had free access to pellet food (San Bolagen Malmo) and tap water Anesthesia was induced with divinyl ether the animals were tracheotomized and immobilized and then maintained on 70 % N_2O and 30 % O_2 In *the metabolite series* a femoral artery was cannulated for blood pressure recording and for anaerobic sampling of blood The body temperature as measured in the rectum was maintained close to 37 °C and the animals were kept at arterial CO_2 tensions of 35—40 mm Hg After a steady state period of 15—30 min the oxygen concentration of the insufflated gas mixture was reduced so as to give arterial O_2 tensions of [illegible]0—22 mm Hg and simultaneously the right common carotid artery was occluded with a rubber-coated clamp The animals were then maintained in this state for another 30 min During this period it was controlled that the P_O remained stable within ±10 % and that the blood pressure did not fall below 120 mm Hg If this occurred the animal was excluded from the material At the end of the 30 min period the brain was frozen *in situ* using the method previously described from the laboratory (Ponten *et al* 1973) The brains were then chiselled out in the frozen state and stored at −80 °C until dissected and analysed (see below)

The rCBF measurements were performed on separate animals in which both femoral arteries and veins were cannulated For measuring rCBF 1 ml of physiological saline containing about 1[illegible] µCi of ^{14}C ethanol was infused *i v* during 30 s with repeated sampling of arterial blood from a 1—2 cm long catheter in one femoral artery At the end of the 30 s period saturated KCl was rapidly injected *i v* to induce cardiac standstill and the animal was decapitated The head was immediately frozen in liquid nitrogen and stored at −80 °C for subsequent counting of the ^{14}C activity together with the blood samples Measurements of rCBF were made both after a 30 min period of hypoxia and unilateral carotid artery clamping and after an additional 30 min recovery period In these latter animals the arterial P_O was brought back to normal and the carotid clamp was removed after the initial 30 min period of hypoxia ischemia

In both the rCBF and in the metabolite series the brains were dissected at subzero temperatures −12 and −2[illegible] °C respectively) Cortical tissue was dissected from both hemispheres to present the areas supplied by the middle cerbral artery ("MCA") and a region containing the boundary zone between the distribution territories of the anterior and the middle cerebral arteries ("BZ" = boundary zone) In the rCBF series additional areas were also sampled to represent the brain stem (from the superior colliculus to the midpons region) the cerebellum the hippocampus and the frontal and occipital poles. The "hippocampus" region could be only grossly dissected and the samples contained some surrounding struc



Series	Clamped normoxia	Clamped hypoxia
n	10	10
Pa_{O_2} mmHg	134 ± 4	205 ± 05
$PaCO_2$ mmHg	38 9 ± 0 7	32 8 ± 1 2
pH	7381 ± 0006	7204 ± 0023
MABP mmHg	155 ± 4	128 ± 2
Temp C	371 ± 01	36 8 ± 01

Fig 1 Cortical regions sampled for regional metabolite studies MCA = middle cerebral artery territory BZ = boundary zone between MCA and the anterior cerebral artery Below the figure are given the physiological parameters in the normoxic and hypoxic groups Values are means ± S E n = number of experiments

tures In the rCBF series the weighed samples were added to vials for subsequent scintillation counting In the metabolite series the tissue was homogenized in HCl methanol at − 2? C and subsequently in perchloric acid for further fluorometric enzymatic analyses

pH P_{CO_2} and P_O in arterial blood were measured with conventional microelectrodes. rCBF was derived from the ^{14}C activity in arterial blood and tissue using a modification of the method described by Landau *et al* (1955) and Reivich *et al* (1969) The modifications included the use of ^{14}C ethanol and a 30 s infusion period omission of a correction for catheter smearing and direct scintillation counting of the ^{14}C activity *in the tissue* (*Ekl* *et al* 1974) Since the tissues sampled could not be considered homogeneous with regard to blood flow and since even ^{14}C-ethanol probably shows some diffusion limitation the values given should be considered as semiquantitative estimates of rCBF (see Discussion)

The following metabolites were measured on the tissue samples glucose glucose-6 phosphate pyruvate lactate α ketoglutarate (α KG) phosphocreatine (PCr) creatine (Cr) ATP ADP and AMP The methods were those of Lowry and Passonneau (1972 for analytical details see Folbergrová *et al* 1972 a and b)

Statistical differences were evaluated with Student s t test using either pooled values for one group or paired values for each animal

Results

A *Regional metabolites*

As stated above cortical tissue representing the distribution territory of the middle cerebral artery as well as the boundary zone between the anterior and middle cerebral arteries, was sampled from both hemispheres Fig 1 indicates the sampling sites and illustrates the physiological parameters in the normoxic and hypoxic groups

TABLE I Regional brain tissue concentrations of lactate (La) phosphocreatine (PCr) creatine (Cr) ATP ADP AMP and energy charge potentials in control and hypoxemic rats Values are means ± S E (μmol g^{-1} of wet tissue) Statistical differences from the appropriate controls are given as $p < 0.05$ = * $p < 0.01$ = ** and $p < 0.001$ = ***

The ECP values were calculated as $\frac{ATP + 0.5\ ADP}{ATP + ADP + AMP}$

	Control (Pooled values)	Hypoxia Intact side MCA	BZ	Clamped side BZ	MCA
La	1.64 ±0.07	10.72*** ±0.87	13.66*** ±0.99	26.53*** ±3.52	32.80* * ±3.83
PCr	4.74 ±0.04	3.44* * ±0.17	3.26* ±0.12	1.85*** ±0.43	1.12* * ±0.37
Cr	5.84 ±0.11	6.88* ±0.10	7.80 ±0.15	9.10* ±0.55	9.33* * ±0.60
ATP	2.97 ±0.02	2.90 ±0.05	2.96 ±0.04	1.91* ±0.30	1.56** ±0.34
ADP	0.289 ±0.005	0.350* ±0.024	0.390* ±0.078	0.739** ±0.124	0.797*** ±0.112
AMP	0.036 ±0.001	0.046 ±0.008	0.057* ±0.008	0.554** ±0.171	0.786 ±0.203
ECP	0.946 ±0.001	0.933 ±0.006	0.976* ±0.006	0.700* ±0.073	0.609** ±0.083

In both the control and the hypoxic animals the arterial P_O P_{CO_2} and pH were very similar to those reported perviously (Salford Plum and Siesjo 1973) In the hypoxic animals the arterial P_O was reduced to 20—21 mm Hg

In the control animals (normoxic but with the right common carotid artery clamped) cortical tissue was sampled bilaterally from the MCA and BZ regions Since there were no regional differences in any of the biochemical parameters measured in the control animals the following tables only show pooled control values for all the cortical sites However statistical differences between control and hypoxic brains were calculated using the values obtained for each sampling site. In the tables the values pertaining to the 4 sites have been arranged (from left to right) to correspond to the order shown in Fig 1

Table I illustrates the values for lactate PCr Cr ATP ADP and AMP as well as for the adenylate energy charge potential (ECP) In the intact hemisphere of the hypoxic animals the MCA region showed marked increases in lactate highly significant decreases in PCr and increases in Cr a moderate increase in ADP but no significant changes in ATP or AMP In the BZ area these changes were somewhat more pronounced and there was also a significant rise in AMP With the exception of the rise in AMP all these changes are typical of those occurring in pure hypoxia i e they are seen in animals that are subjected to a decrease in arterial P_{O_2} but which have intact carotid arteries and a blood pressure of 120 mm Hg or more (see Discussion)

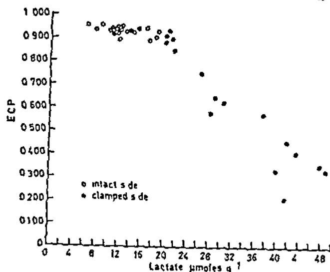


Fig 2 The relation between lactate (La) and energy charge potential (ECP) in cortical regions from the hemisphere on the intact side (*open circles*) and on the carotid artery clamped side (solid circles)

On the clamped side there was a massive lactic acidosis and changes in organic phosphates that suggest a major derangement of the energy state These changes were similar to but somewhat more pronounced than those reported previously (Salford Plum and Siesjo 1973) However the changes were more marked in the MCA than in the BZ region Thus the MCA region showed a significantly higher lactate concentration ($p < 0.05$) lower PCr and ATP ($p < 0.05$) a higher AMP concentration ($p < 0.05$) and accordingly a lower adenylate energy charge ($p < 0.01$) Fig 2 shows that in many cortical samples (especially in the MCA region) the lactate concentration exceeded 25 μmol g^{-1} and further that there was a linear relationship between the increase in lactate concentration and the decrease in adenylate energy charge This relationship was particularly evident at lactate concentrations above 25 μmol g^{-1}

TABLE II Regional brain tissue concentrations of glucose glucose 6 phosphate (G 6 P) pyruvate and α ketoglutarate (α KG) in control and hypoxemic rats Values are means ± S E (μmol g^{-1} of wet tissue) Statistical differences from the appropriate controls are given as $p < 0.05$ = * $p < 0.01$ = ** and $p < 0.001$ = ***

	Control animals (Pooled values)	Hypoxia Intact side MCA	BZ	Clamped side BZ	MCA
Glucose	5 10 ±0 24	6 76 ±0 34	7 32* ±0 31	5 08 ±1 47	4 40 ±1 64
G-6-P	0 112 ±0 005	0 138* ±0 005	0 163** ±0 009	0 218** ±0 022	0 211** ±0 010
Pyruvate	0 114 ±0 003	0 301** ±0 015	0 345* ±0 013	0 210* ±0 011	0 1[illegible] ±0 011
α KG	0 126 ±0 005	0 153* ±0 011	0 179 ±0 002	0 0[illegible]9* ±0 0[illegible]1	0 049 ±0 074

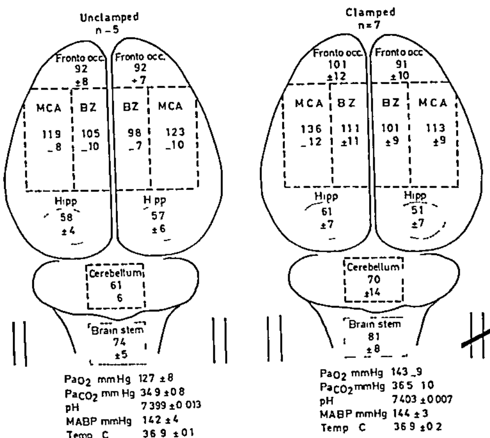


Fig 3 Sampling sites for measurements of reg onal cerebral blood flow in unclamped and carotid artery clamped normoxic animals Fronto occ = pooled frontal and occipital grey and white tissue MCA = middle cerebral artery territory BZ = boundary zone between MCA and the anterior cerebral artery Hipp = h ppocampus n = number of experiments Values are ± S E (ml (100 g)$^{-1}$ min^{-1}) The physiological parameters are given below the figure

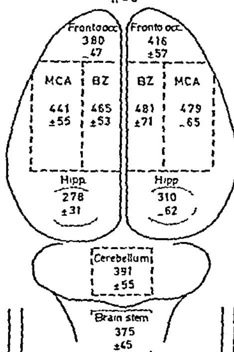
Table II illustrates the tissue concentrations of glucose G 6 P pyruvate and α ketoglutarate On the intact side the MCA region showed significant increases in G 6 P pyruvate and α KG while the BZ region had a small increase in glucose as well On the clamped side the glucose concentration was not altered from the normal and there were highly significant increases in G 6 P Only the BZ region showed an increase in pyruvate and both regions had reduced concentrations of α KG Since none of the regions showed decreases below the normal in the concentrations of the substrates glucose G 6 P or pyruvate the results seem to exclude the presence of ischemia of a degree sufficient to limit substrate supply (see Discussion)

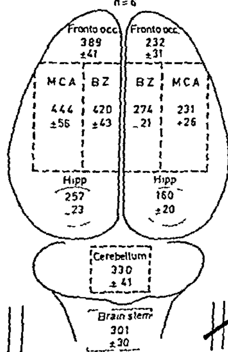
HYPOXIA

Pa_{O_2} 22

Unclamped n = 6

Clamped n = 6





	Unclamped		Clamped
Pa_{O_2} mmHg	21.9 ± 0.3	Pa_{O_2} mmHg	21.2 ± 0.5
Pa_{CO_2} mmHg	29.7 ± 1.0	Pa_{CO_2} mmHg	30.2 ± 1.1
pH	7.268 ± 0.030	pH	7.213 ± 0.034
MABP mmHg	131 ± 3	MABP mmHg	144 ± 5
Temp C	36.9 ± 0.1	Temp C	37.1 ± 0.1

Fig 4 Regional cerebral blood flow in unclamped and carotid artery-clamped hypoxic animals n = number of experiments Values are means ± S E (ml (100 g)$^{-1}$ min^{-1}) The physiological parameters are given below the figure

B *Regional CBF*

The main objective of the rCBF measurements was to evaluate the rate of blood flow in the BZ and the MCA regions in animals with unilateral carotid artery ligation either after 30 min of combined hypoxia and carotid artery clamping or following an additional 30 min recovery period of restituted carotid artery flow and arterial P_{O_2} However in order to obtain more information on rCBF in the brain under the present experimental conditions flow values were also derived for other hemispheric regions (the frontal and occipital poles and the hippocampus) as well as for two midline structures (cerebellum and brain stem) The fronto-occipital parts were chosen since they include both grey and white matter and the hippocampus region was sampled since this area shows early histopathologic changes with the present model of combined hypoxia and (relative) ischemia The cerebellum

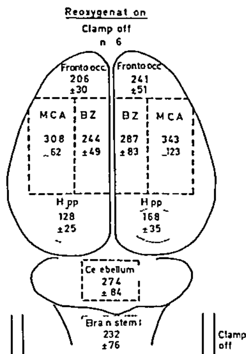


Fig 5 Regional cerebral blood flow at the end of a 30 min recovery period following 30 min of hypoxia (Pao 22 mm Hg) and unilateral carotid artery occlusion At the end of the hypoxic ischemic period the carotid clamp was removed and the Pao was brought back to normal (> 100 mm Hg) n = number of experiments Values are means ± S E (ml (100 g)⁻¹ min)

and brain stem were studied in order to evaluate if the clamping procedure reduces CBF in structures that are predominantly supplied from the vertebral system For animal that were clamped whether normoxic or hypoxic the rCBF values obtained were compared to those measured in normoxic or hypoxic animals with an intact carotid artery circulation (see MacMillan *et al* 1974)

Fig 3 shows the rCBF values obtained in normoxic animals (unclamped and clamped) In the unclamped animals all identical structures had similar rCBF values ($p > 0\,05$) However a statistical analysis on paired samples showed that the rCBF was higher in the MCA than in the BZ area ($p < 0\,01$) The figure demonstrates that the frontal and occipital poles which contained also white matter had rCBF values that were only slightly lower than those of the pure cortical structures However the hippocampal cerebellar and brain stem structures had lower rCBF values than the cerebral cortical structures (see Discussion)

In the clamped animals the rCBF values obtained on the intact side did not differ significantly from those measured in the unclamped animals However in all cerebral regions the rCBF values on the clamped side were somewhat lower than on the unclamped side demonstrating that the clamping procedure reduced flow on the ipsilateral side *e g* rCBF in the MCA region on the clamped side was statistically different ($p < 0\,01$ paired values) from the rCBF in the MCA region on the unclamped side However this small reduction in rCBF did not affect the metabolic pattern of the MCA and BZ regions (see above)

Fig 4 illustrates the rCBF values in hypoxic animals without and with a carotid clamp In the unclamped animals there was a 4 to 6 fold increase in rCBF (MacMillan Salford and Siesjo 1974) In the clamped animals the intact side showed increases in rCBF that did not significantly deviate from the corresponding valus obtained in the hypoxic animals with intact carotid arteries In the cerebellum and brain stem the clamped animals had somewhat lower mean rCBF values than the unclamped animals but the differences were not statistically significant ($p > 0\ 05$) On the clamped side all structures showed a less pronounced increase in rCBF, and in the MCA region the rCBF was only about half of that measured on the intact side ($p < 0\ 01$) However, in all structures on the clamped side the rCBF values were still significantly increased above the normal ($p < 0\ 001$) Thus although the clamping procedure limited the increase in rCBF following hypoxia the data demonstrate that hyperemia still occurred and that absolute ischemia was not present

Fig 5 shows the rCBF values measured 30 min after the arterial P_O had been brought back to normal, and the carotid clamp had been removed The values showed a considerable scatter some being close to normal (*cf* Fig 2) and others 3—4 times higher than normal However in none of the animals were there decreases in rCBF below the normal The results seem to exclude the possibility that absolute ischemia developed in the 30 min period following the hypoxic insult A few animals in the hypoxic clamped group were examined with autoradiography (Reivich *et al* 1969) in order to visualize the differences in flow between the clamped and the unclamped side The medial part of the BZ of the clamped side had a high flow probably due to contribution through the anterior cerebral artery This effect is also shown in Fig 4 where the BZ has a higher flow than the MCA in the clamped side

Discussion

The present experiments have confirmed our previous observation that if the arterial P_O is reduced to about 20 mm Hg in animals which have one carotid artery clamped the cerebral cortex on the clamped side shows a major derangement in energy metabolism in spite of the fact that levels of carbohydrate substrates such as glucose, G 6 P and pyruvate remain normal or elevated This suggests that the failure of energy metabolism is not due to ischemia *i e* to a reduction of rCBF below normal an assumption which is born out by the rCBF measurements which showed that the flow on the clamped side was considerably higher than in the controls In discussing these results we will make two assumptions that appear reasonable (1) that the tissue samples used to measure metabolites or rCBF do not contain regions of absolute ischemia and (2) that since the experimental conditions in the series used for studying rCBF metabolites and histopathology respectively were identical inferences can be drawn by comparing the results of one series to those of the others If these assumptions are valid we can conclude that hypoxia

alone (i e a reduction in oxygen delivery without an absolute decrease in CBF) can lead to irreversible neuronal damage Since this conclusion has a direct bearing on the pathophysiology of anoxic brain damage it seems warranted to discuss the various factors that may cause such damage in conditions of ischemia or hypoxia

It is generally assumed that the development of irreversible anoxic damage to neurons can be related to the severity and the duration of the tissue hypoxia possibly also to the retention of potnetially toxic waste products such as lactic acid and ammonia Theoretically we could therefore assume that instantaneous total ischemia should represent a maximally harmful situation since it quickly leads to consumption of the slender stores of oxygen and prevents waste products to leave the tissue In the rat cerebral cortex total ischemia leads to energy depletion within 3—5 min the lactate concentration increases to 13—14 μmol g^{-1} within 1—3 min and there is a gradual increase in ammonia to values about 5 times the normal (Ljunggren *et al* 1974 a) In view of these pronounced metabolic changes it is surprising that energy metabolism may be restituted even after prolonged ischemic periods Thus in the rat cerebral cortex the adenylate energy charge returns to within 1—2 % of normal and the accumulated lactate and ammonia disappear if the circulation is restored after a 15 min ischemic period (Ljunggren *et al* 1974 b) With this model ischemic periods of longer than 15 min cannot be studied However using another model in the cat Hossman and Sato (1970 a and b) noted recovery of EEG and of evoked cortical responses even after 60 min of total ischemia and Hinzen *et al* (1972) found recovery of energy metabolism in the isolated dog s head after 30 min of circulatory arrest Although histopathological studies are not available to show whether or not neuronal damage is present in these preparations the revival of neuronal activity and meabolism observed speaks against gross neuronal damage

The results obtained in *total* ischemia should be considered in relation to those obtained in *partial* ischemia and to those obtained in the present material Partial ischemia induced by a lowering of the mean arterial blood pressure to 25 mm Hg induces irreversible neuronal damage even if the hypotension is limited in duration to 15 min (Brierley *et al* 1969) Thus provided that these experiments can be directly compared to those described above the results lead to the paradoxical conclusion that partial ischemia is more harmful than total ischemia In the present model the combination of arterial hypoxia and unilateral carotid artery ligation for 30 min leads to a major derangement of energy metabolism in the hemisphere on the side of the ligation When such animals are studied after an additional recovery period of 30 min some show a persisting derangement of energy state and many exhibit histopathologic alterations in the form of microvacuolation presumably representing swollen mitochondria (see Brown and Brierley 1972) and ischemic cell changes (Salford Plum and Brierley 1973) If it is assumed that the mitochondrial damage occurs during the first 30 min of hypoxia ischemia this may explain why the energy state of the tissue is not restituted in spite of the fact that the animal is reoxygenated and the carotid artery clamp is removed How

ever we are then bound to conclude that such damage occurs in 30 min in spite of the fact that a maintained circulation carries some oxygen to the tissue and theoretically, allows waste products to be removed

Admittedly the experiments performed on total and partial ischemia as well as on hypoxia combined with relative ischemia, are sufficiently dissimilar to preclude rigid conclusions but the results nevertheless indicate that partial ischemia, and hypoxia combined with relative ischemia, may involve adverse effects that do not accompany total ischemia These effects may possibly be related to the lactic acidosis accompanying a continued supply of substrate Thus in total ischemia the lactic acidosis is determined by the carbohydrate stores of the tissue (glucose and glycogen) In rats under nitrous oxide anesthesia these stores suffice to increase the lactate levels to 13—15 μmol g^{-1} during total ischemia (Ljunggren *et al* 1974 a) and even if the animals are rendered grossly hyperglycemic the lactate content does not increase above 21 μmol g^{-1} (Ljunggren *et al* in preparation) In contrast the present model with hypoxia and relative ischemia may give rise to tissue lactate levels as high as 30—50 μmol g^{-1} Fig 2 indicates a clear relationship between increase in lactate and decrease in adenylate energy charge at lactate values exceeding 25 μmol g^{-1} Admittedly such a relationship could mean that in severely hypoxic tissues there is major derangements in ATP ADP and AMP which in the presence of a substrate supply allows excessive amounts of lactic acid to be formed However it cannot be excluded that the lactic acidosis when excessive is in some way the cause of the derangement in energy state Theoretically the severe acidosis associated with such an accumulation of lactate could affect the tissue adversely either by interfering with the capillary circulation or by facilitating autolytic processes In the present experiments measurements of the rCBF at the end of the hypoxic ischemic period or following a 30 min recovery period gave no evidence of absolute ischemia Thus provided that the samples used for measuring rCBF were not grossly inhomogeneous in capillary circulation it can be tentatively assumed that the acidosis affected the tissue directly It may thus be that a continued substrate supply to a severly hypoxic tissue adversely affects its viability via other mechanisms than those influencing the circulation (*cf* Hossman and Kleihues 1973)

In summary the present results have shown that the relative ischemia induced by carotid artery ligation in hypoxic animals exaggerates the cellular hypoxia without limiting substrate supply to the hypoxic cells As a consequence the energy state fails and massive amounts of lactic acid accumulate in the tissue The resulting acidosis is more pronounced than that observed in total ischemia We have suggested that the acidosis adversely affects the tissue via its effect on autolytic processes The detailed mechanisms of such a hypothetical effect are entirely unknown and the proposed coupling between acidosis and cell damage is speculative However the pathophysiological factors underlying anoxic nerve cell damage are sufficiently important to warrant serious consideration of all potentially contributing factors

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References

BRIERLEY J B A W BROWN B J EXCELL and B S MELDRUM Brain damage in the rhesus monkey resulting from profound arterial hypotension I Its nature distribution and general physiological correlates *Brain Res* 1969 *13* 68—100

BROWN A W and J B BRIERLEY Anoxic ischaemic cell change in rat brain Light microscopic and fine structural observations *J Neurol Sci* 1972 *16* 59—84

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FOLBERGROVA J V MACMILLAN and B K SIESJÖ The effect of moderate and marked hypercapnia upon the energy state and upon the cytoplasmatic NADH/NAD ratio of the rat brain *J Neurochem* 1972 a. *19* 2497—2505

FOLBERGROVA J V MACMILLAN and B K SIESJO The effect of hypercapnic acidosis upon some glycolytic and Krebs cycle associated intermediates in the rat brain. *J Neurochem* 1972 b *19* 2507—2517

HINZEN D H U MULLER, P SOBOTKA E GEBERT R. LANG and H HIRSCH Metabolism and function of dog s brain recovering from longtime ischemia *Amer J Physiol* 1972 *223* 1158—1164

HOSSMAN K A and P KLEIHUES Reversibility of ischemic brain damage *Arch Neurol* (Chic) 1973 *29* 375—384

HOSSMAN K A and K SATO Recovery of neuronal function after prolonged cerebral ischemia. *Science* 1970 a. *168* 375—376

HOSSMAN K A and K SATO The effect of ischemia on sensorimotor cortex of the cat. Electrophysiological biochemical and electronmicroscop cal observations *Z Neurol* 1970 b *198* 33—45

LANDAU W M W H FREYGANG L P ROSLAND L SOKOLOFF and S S KETY The local circulation of the living brain values in the unanesthetized and anesthetized cat. *Trans Amer Neurol Ass* 1955 *80* 125—129

LJUNGGREN B R A RATCHESON and B K SIESJO Cerebral metabolic state following com plete compression ischemia. *Brain Res* 1974 b In press

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LOWRY O H and J V PASSONNEAU *A Flexible System of Enzymatic Analysis* Academic Press New York and London 1972

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PONTEN U R. A RATCHESON L G SALFORD and B K SIESJO Optimal freezing condi tions for cerebral metabolites in rats *J Neurochem* 1973 *1* 1127—1138

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SALFORD L G F PLUM and J B BRIERLEY Graded hypoxia-oligemia in rat brain II Neuropathological alterations and their implications *Arch Neurol* (Chic) 1973 b *29* 234—238

SALFORD L G F PLUM and B K SIESJO Graded hypoxia-oligemia in rat brain I Biochemical alterations and their implications *Arch Neurol* (Chic.) 1973 a *29* 227—233

SIESJO B K and L NILSSON The influence of arterial hypoxemia upon labile phosphates and upon extracellular and intracellular lactate and pyruvate concentrations in the rat brain. *Scand J clin Lab Invest* 1971 *27* 83—?

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From the Brain Research Laboratory E blocket University Hospital Lund Sweden

Measurement of Overall Blood Flow and Oxygen Consumption in the Rat Brain

By

BENGT NILSSON

A method has recently been developed for quantitative measurement of cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMR_{O_2}) in rats (Eklof *et al* 1973 Norberg and Siesjö 1974) The method, which is a modification of the Kety and Schmidt (1948) technique for CBF measurement, involves saturation of the animal with ^{133}Xenon for 20 min followed by repeated sampling of arterial and venous blood during the desaturation period With this method CMR_{O_2} in rat brain has been found to approximate 10 ml (100 g)$^{-1}$ min^{-1} a value about 3 times as high as that of the human brain There are 2 possible explanations for this (1) Since the measurements in rats are based on venous blood from the superior sagittal sinus the variation is explained by differences in CMR_{O_2} between cortical grey matter (rat) and whole brain (man) (2) There are true species differences possibly reflecting an inverse relationship between body size and CMR_{O_2} To settle this question a modification of the CBF method has been employed permitting measurement of CBF and CMR_{O_2} in whole rat brain

The experiments were carried out on unfasted male Wistar rats (300–400 g) that were maintained paralyzed and artificially ventilated on 70 % N_2O and 30 % O_2 Arterial P_{CO_2} was adjusted close to 35 mm Hg and body temperature was maintained at 37 °C CBF and CMR_{O_2} were determined as described by Eklof *et al* (1973) and Norberg and Siesjo (1974) However in order to sample venous blood representative of the whole brain the extracranial portion of the transverse sinus was dissected free at the retroglenoid foramen and cannulated with the tip of the cannula pushed towards the bony canal In this region the transverse sinus being the main venous outflow of the brain receives blood not only from cortical tissue via the superior sagittal sinus but also from central lateral and basal parts of the forebrain from dorsal parts of the cerebellum and possibly also from upper brain stem regions (Greene 1959) The length of cannula used was 2–3 cm Blood from the cannula was allowed to flow directly into glass capillaries either without or during transient compression of the contralateral transverse sinus The general procedure was to administer ^{133}Xe in the inhaled gas mixture for 10–30 min (saturation) and to sample arterial and cerebral venous blood repeatedly for measurement of ^{133}Xe activity and oxygen content (T_{O_2}) during the desaturation *CBF was calculated from the desaturation activity curves* according to the trapezoid rule and using a partition coefficient (λ) of 0.82 CMR_{O_2} was obtained by multiplying the CBF with the arteriovenous difference in T_{O_2} In some experiments the animal was decapitated and the head was immediately frozen in liquid nitrogen at the end of the desaturation period Samples of tissue from various regions were chiselled out under liquid nitrogen and analyzed for activity of ^{133}Xe

Irrespective of the length of the saturation period *(10–30 min) the activity* of ^{133}Xe in brain tissue approached that of the arterial blood after 15 min of desaturation

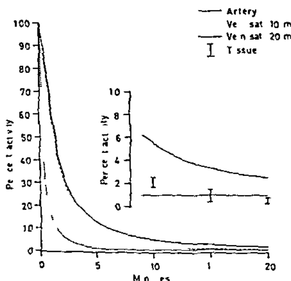


Fig 1 Representative Xe activity curves of arterial and venous blood during desaturation following saturation periods of 10 and 20 min The venous blood was sampled without compression of the contralateral transverse sinus The late parts of the curves are shown magnified and compared to activity of brain tissue (range) taken at 10 15 and 20 min of desaturation The curves were drawn as the best visual fits to the experimental points

ndicating that all brain regions equilibrated with arterial blood during that period In contrast various specimens of extracerebral tissue from the head (skin bone muscle) showed considerable amounts of remaining activity Thus following a 20 min saturation period the extracerebral tissue samples taken at 15 min of desaturation still showed 10–20 % of the maximal saturation activity of blood

In the beginning of the present series venous samples taken without compression of the contralateral transverse sinus showed a prolonged tail This varied with the length of the saturation period (Fig 1) thus indicating extracerebral contamination of the venous blood sampled The blood flow value calculated from these curves varied between 40 and 60 ml (100 g)$^{-1}$ min^{-1} and the metabolic rate for oxygen was 3–4 ml (100 g)$^{-1}$ min^{-1}

Subsequently with additional attention to the positioning of the catheter with high ligation and careful fixation and compression of the contralateral transverse sinus during sampling the venous activity of ^{133}Xe was found to approach that of the arterial blood (and brain tissue) at 15 min of desaturation (Fig 2) Since this level of venous activity was obtained despite considerable ^{133}Xe activity in extracerebral tissue contamination of the venous blood should have been minimal At a Pa_{CO_2} of 35 6±1 0 mm Hg (Mean±S E) the CBF thus determined was 79 2±2 8 ml (100 g)$^{-1}$ min^{-1} and the CMR_{O_2} was 7 53±0 20 ml (100 g)$^{-1}$ min^{-1}

The results of the venous sampling emphasize the need to exclude extracerebral contamination When such precautions were taken the CMR_{O_2} of the whole brain was found to be about 75 % of the corresponding cortical values (see Norberg and Siesjo 1974) This value is still at least twice the value of the human brain indicating appreciable species differences probably reflecting and inverse relationship between body size and CMR

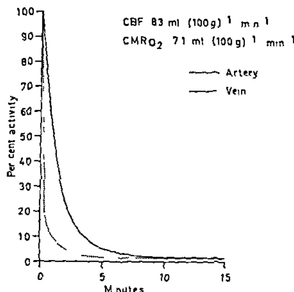


Fig 2 Representative desaturation curves for ¹³³Xe with careful positioning of the venous catheter and compression of the contralateral transverse sinus The tissue was saturated with ¹³³Xe for 20 min before desaturation

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References

Eklof B N A Lassen L Nilsson K Norberg and B K Siesjo Blood flow and metabolic rate for oxygen in the cerebral cortex of the rat *Acta physiol scand* 1973 *88* 587—589

Greene E Ch *Anatomy of the rat* 1959 Hafner Publ Co New York

Kety S S and C F Schmidt The nitrous oxide method for the quantitative determination of cerebral blood flow in man theory procedure and normal values *J clin Invest* 1948 *27* 484—492

Norberg K and B K Siesjö Quantitative measurement of blood flow and oxygen consumption in the rat brain *Acta physiol scand* 1974 *91* 154—164

Acta physiol scand 1974 92 145—155

From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

The Influence of Sympathetic Activity and Histamine on the Blood-Tissue Exchange of Solutes in Canine Adipose Tissue

By

Birgitta Linde Guy Chisolm[1] and Sune Rosell

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Abstract

Linde B G Chisolm and S Rosell *The influence of sympathetic activity and histamine on the blood tissue exchange of solutes in canine adipose tissue* Acta physiol scand 1974 92 145—155

By means of a single injection technique diffusion capacities for ^{14}C-sucrose and ^{3}H polyethylene glycol (M W 800—1000) (PEG) expressed as the PS product of Renkin were determined in dog subcutaneous adipose tissue

Under resting conditions PS for sucrose and PEG were approximately 2 and 1 $ml \times min^{-1} \times 100\ g^{-1}$ respectively During maximal vasodilatation induced by prostaglandin E_2 (PGE_2) and papaverine the PS products were approximately doubled The effects of sympathetic nerve stimulation (1—6 Hz) and histamine on the diffusion capacities were compared with controls at the same blood flow During sympathetic nerve stimulation PS for sucrose increased 15 % in spite of a vasoconstriction When superimposed on a maximal vasodilatation sympathetic nerve stimulation induced increases in PS for sucrose of approximately 40 % and in PS for PEG of about 20 % Histamine increased PS for sucrose 40 % in a dosedependent fashion compared with a vasodilatation of the same magnitude induced by papaverine or PGE_2 The results indicate that sympathetic nerve stimulation causes an increase in vascular permeability for solutes in canine subcutaneous adipose tissue Histamine seems to have the same effect

In a previous study on canine subcutaneous adipose tissue Öberg and Rosell (1967) found that the capillary filtration coefficient (CFC) increased significantly upon stimulation of the sympathetic nerve supply This increase in CFC occurs in spite of an unchanged or even a decreased exchange area as judged from the rate of disappearance of ^{133}Xe or $^{1[illegible]3}I$ from a local depot (Linde and Gainer 1974) Thus it appears that sympathetic stimulation while decreasing the surface area for exchange may increase the permeability

In another study the isovolumetric capillary pressure (P_{ci}) was found to decrease during sympathetic nerve stimulation This finding is consistent with the proposed increase in vascular permeability provided that the permeability change

[1] Present address Dept. of Chemical Engineering M I T Cambridge Mass 02139 USA

is of such a nature that it permits the passage of plasma borne, osmotically active substances (Rosell *et al* 1974)

The object of the present investigation was to further examine possible changes in vascular wall permeability in subcutaneous adipose tissue during sympathetic nerve stimulation by using the single injection technique described by Chinard (1955) and Crone (1963) Provided that the blood flow is above the flow limited range this method allows the calculation of the diffusion capacity of the vascular bed for molecules that pass from the vessels into the tissue

Method

Female dogs anesthetized with sodium penthobarbital (30 mg/kg b wt with supplement when necessary) were used for the experiments Tracheostomies were performed on all animals Arterial blood gases were analyzed in most animals and if necessary artificial positive pressure breathing was instituted to maintain normal blood gas values The subcutaneous adipose tissue preparation in the inguinal region as described by Rosell (1966) was used The tissue was freed from all surrounding tissues including the skin except for the main artery vein and nerve Blood flow was measured with the aid of a silicone filled drop counter inserted into the arterial cannula and was continuously monitored on a Grass Polygraph on which the arterial blood pressure was also recorded Venous samples were collected from a 10 to 15 cm long cannula inserted into the vein Care was taken to adjust the opening of this cannula to a level where no interference with the blood flow was seen Stimulation of the nerve was performed via bipolar silver electrodes with square wave impulses from a Grass stimulator The stimulation parameters were 0 5 to 6 Hz 2 ms duration and 8 to 12 V The temperature of the preparation was kept at 37—38 C with the aid of a heating lamp To preserve moisture the tissue was covered with a thin plastic sheet and saline soaked gauze pads

The single injection technique (Chinard 1955 Crone 1963) was used Single rapid injections of a high molecular weight reference substance and a diffusible test substance were made into the arterial supply to the adipose tissue The concentration versus time curves for the individual substances normalized with respect to the concentration in the fluid injected were then determined in the venous outflow The fractional extraction of the test substance was calculated using the assumption that the reference substance does not penetrate the vascular wall to any significant extent in a single passage

Radio-iodinated human serum albumin ^{131}I (AB Atomenergi Studsvik) was used as a reference substance and ^{14}C-sucrose (M W 342) (The Radiochemical Centre Amersham) and ^{3}H polyethylene glycol PEG (M W 800—1000) (New England Nuclear Boston) as test substances The radioactive compounds were given in amounts of 0 5 to 5 μCi per injection. The PEG was filtered through a Diaflo membrane UM 05 (Amicon) which retains substances with a M W above 500 Only 1—2 % of the radioactivity passed trough this filter The labelled substances were mixed with serum and if necessary concentrated albumin solution to adjust the colloid osmotic pressure to that of blood Appropriate amounts of carrier substances were added to the labelled compounds

Before each injection aliquots of the injection fluids were pipetted into ten separate vials for scintillation counting to get a reliable measure of the concentrations of the substances injected Venous hematocrit determinations were performed before each injection. 25 to 100 μl of the injection fluid were injected during approximately 1 s with a Hamilton syringe via rubber tubing inserted into the arterial inflow cannula. Venous samples were collected dropwise usually on a cold siliconized glass plate and immediately pipetted into the vials used for counting In some cases the drops were collected directly into the vials The sampling was continued for 30 s to 2 min after the injection Each sample containing 50 μl of whole blood was prepared for liquid scintillation counting A Packard Tri Carb 3375 liquid scintillation spectrometer was used for the counting The data from the liquid scintillation counter were corrected for background overhearing and quenching using appropriat blanks and standards Extraction values were calculated for each separate sample using the equation

$$E = \frac{C_{ref}-C_{test}}{C_{ref}}$$

where C_{ref} and C_{test} are the counts per min of the reference and test substance normalized by their concentrations in the fluid injected One extraction value for each injection was also calculated according to the area method in which integration was performed over the time interval until the separate extraction values started to decrease As an indication of e peri

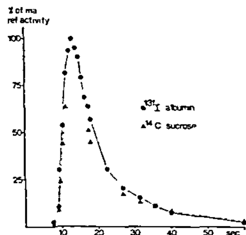


Fig 1 Canine subcutaneous adipose tissue Venous concetration time curves for ^{131}I albumin and ^{14}C-sucrose during control conditions Plasma flow = 18 ml × min^{-1} × 100 g^{-1} Tissue weight = 28 g Exp 730425

mental reproducibility duplicate determinations of the area extraction within the same experiment gave a coefficient of variation of 1 4 extraction % (n = 10) A clearance value (extraction × flow) was calculated for each injection using the area extraction and the plasma flow Q_{pl} (since all labelled substances were suspended in the plasma phase) Plasma flow = blood flow × (1 − Hct) Likewise a PS-product, permeability—surface area product, (Renkin 1959) was calculated according to the expression $PS = -Q_{pl} \times \ln (1-E)$ where P is the permeability coefficient of the diffusion barrier and S its surface area. All calculations were made by digital computer

The main purpose of the present study was to record changes in diffusion exchange between control and nerve stimulation conditions For this purpose it was necessary to determine the relative importance of blood flow and diffusion capacity for diffusion exchange *i e* at what levels of blood flow sucrose and PEG were flow and diffusion limited respectively As seen in Fig 2 clearance for sucrose appears to be largely flow dependent during resting conditions. Vasodilating agents prostaglandin E_2 (PGE_2) and papaverine were therefore administered in an attempt to reach blood flow levels where clearance was predominantly diffusion limited. Infusions of the vasodilators were started before a control run and were continued throughout the experiment, *i e* during 4 to 7 injections. Even if the transcapillary diffusion appeared largely diffusion limited at the higher blood flow levels it seemed important to keep blood flow at the same level under control and stimulated conditions This was accomplished in different ways At low stimulation frequencies *e g* 0.5—1 5 Hz, not much interference with the blood flow was encountered in contrast to the situation at higher frequencies The constriction occurring at the higher frequencies always subsided with time and had often vanished 10 to 15 min after the onset of stimulation In those cases where blood flow did not reach the control level spontaneuosly elevation of the blood pressure was accomplished by carotid occlusion In two experiments a constant flow perfusion method (Renkin 1959) was used to obtain the same flow level during control and nerve stimulation periods

The material was divided into 2 groups on the basis of whether the resistance during stimulation was elevated or not. Thus one group consisted of determinations performed when the arterial resistance was elevated *i e* when the vasoconstriction occurring during sympathetic nerve stimulation was still present and blood flow kept at control levels by an elevation of the perfusion pressure (Group I) In the other determinations resistance was at control levels during nerve stimulation (Group II) This was accomplished either by using low stimulation frequencies or by waiting until the vasoconstriction vanished spontaneously

In 8 expts the effect of histamine on the diffusion capacities of sucrose and PEG was examined Histamine dihydrochloride was infused intraarterially The plasma concentrations were calculated to be between $5\ 1 \times 10^{-7}$ and $1\ 1 \times 10^{-5}$ M To obtain the same level of flow prostaglandin E_2 or papaverine were infused as controls

Values are given as means ± standard deviations A t test for paired variates was used for testing differences in PS-products between resting condition and experimental intervention Partial correlation coefficients according to Snedecor and Cochran (1967) were calculated to investigate correlation between increases in PS-products and stimulation frequency Linear regression analysis was used for determining dependency of increases in PS on histamine dose

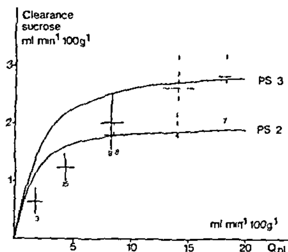


Fig 2 Canine subcutaneous adipose tissue Grouped clearance values for sucrose related to plasma flow (Q_{pl}) The crosses indicate ±S D —|— = resting conditions = no vasodilator agents added –·– = vasodilator agents (papaverine or prostaglandin E_2) added The figures below the crosses indicate the number of determinations The continuous lines show the theoretical clearance flow curves for PS equal to 2 and 3 ml × 100 g⁻¹ No statistical difference in clearance was found between the highest resting group and any of the two higher groups in which vasodilating agents were used

Results

Fig 1 shows typical curves of venous concentration versus time for ^{14}C labelled sucrose (the diffusible tracer) and ^{131}I labelled albumin (the reference tracer) The concentration of sucrose is lower than the concentration of the reference substance until approximately 10 % of the maximal reference activity remains indicating a late onset of back diffusion.

On the basis of the concentrations of the reference and test substances the extraction values were calculated and plotted versus time Different shaped extraction time curves were then obtained Approximately 70 % of the curves were of the type presented in Fig 4 in which there was an initial rise in the extraction followed by a plateau the length of which varied between experiments after which the extraction fell Following the other injections the extractions reached the plateau level from an initially higher value or started directly on the plateau (Fig 5)

Clearance as related to plasma flow

In Fig 2 grouped clearance values for sucrose are plotted against plasma flow Under resting conditions with no vasodilating agents administered the plasma flow varied between 2 and 8 ml / min⁻¹ × 100 g⁻¹ which corresponds to a blood flow between 4 and 15 ml × min⁻¹ / 100 g⁻¹

In the range of flow values studied there was no indication of an upper limit for the clearance of sucrose (Fig 2) (For detailed discussion of clearance flow relationships see Renkin 1959)

By administering vasodilator substances a mean plasma flow of around 17 ml × min⁻¹ / 100 g⁻¹ was obtained This corresponds to a maximal blood flow (Öberg and Rosell 1967) Under these conditions the clearance values for sucrose adapted well to the clearance flow curve for PS equal to 3 ml × min⁻¹ × 100 g⁻¹ (Fig 2) and the transcapillary transport of sucrose appeared largely diffusion limited

In Fig 3 the clearance values for PFG plotted against plasma flow are shown

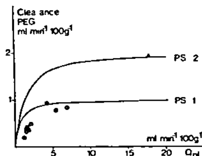


Fig 3 Canine subcutaneous adipose tissue Clearance for polyethylene glycol 800—1000 (PEG) related to plasma flwo (Q_{pl}) O = resting conditions △ = vasodilator agents (papaverine or prostaglandin E_2) added The continuous lines PS equal to 1 and 2 ml×min⁻¹×100 g⁻¹ represent the theoretcial clearance flow curves for

The clearances under resting conditions without vasodilating agents approached the curve for PS equal to 1 ml×min⁻¹×100 g⁻¹ at a plasma flow of around 5 ml×min⁻¹×100 g⁻¹ At maximal vasodilatation the corresponding limiting clearance appeared to be slightly above 2 ml×min⁻¹×100 g⁻¹

Sympathetic nerve stimulation

In Fig 4 the extraction of sucrose under control conditions and during nerve stimulation was plotted versus time It is evident that sympathetic nerve stimulation resulted in an increase in the extraction of sucrose The curve for PEG was the same in principle Table I shows the results of sympathetic nerve stimulation In the group in which plasma flow was kept at control levels by increasing the perfusion pressure (Group I) the increase in PS product for sucrose was approximately 15 % In group II in which resistance was the same during control and stimulation the increase for sucrose and PEG were 40 % and 20 % respectively In the range of stimulation frequencies studied (0 5 to 6 Hz) no significant correlation as tested by partial correlation analysis (Snedecor and Cochran 1967) was found between the increase in PS product and stimulation frequency

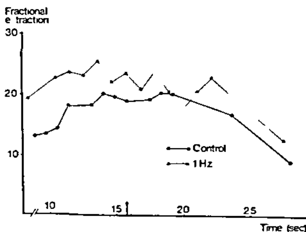


Fig 4 Canine subcutaneous adipose tissue Extraction of ¹⁴C-sucrose plotted versus time during control and during stimulation of sympathetic nerves (1 Hz) Arrows indicate time of maximal reference activity

Tissue weight = 25 g Plasma flow = 18 ml×min⁻¹×100 g⁻¹ Exp 730724

TABLE I

	Fractional extraction		PS ml × min⁻¹ × 100 g⁻¹	
	sucrose	*PEG*	*sucrose*	*PEG*
GROUP I				
Increased R				
Cont	0 25 ± 0 09		2 4 ± 0 8	
Stim	0 28 ± 0 09		2 8 ± 0 9	
	$n = 11$			
P			< 0 001	
GROUP II				
Control R				
Cont	0 17 ± 0 03	0 13 ± 0 01	3 1 ± 0 6	2 0 ± 0 6
Stim	0 22 ± 0 04	0 14 ± 0 02	4 4 ± 1 2	2 3 ± 0 8
	n = 14	n = 9		
P			< 0 001	< 0 05

PS was determined repeatedly during the course of nerve stimulation and was found to remain increased during the stimulation period, the longest periods were around 45 min In the post stimulatory period following stimulation periods averaging 30 min (n = 6) PS for sucrose was found to remain elevated for approximately 30 min

Infusion of histamine

Histamine was infused in amounts causing vasodilatation (5 1 × 10 to 1 1 × 10⁻⁵ M) The increase in the extraction of sucrose is shown in Fig 5 As control PGE or papaverine were infused to induce the same level of plasma flow In all but one determination the PS products for sucrose and PEG were increased The increases in the PS for sucrose were significantly correlated to the logarithm of the histamine concentration (Fig 6)

Discussion

From several studies on transcapillary transport in which single injection techniques have been used it is obvious that the curve of fractional extraction versus time shows considerable variation in shape depending for example on the species and organ studied This variation is in part due to differences in blood vessel geometry non uniformity of flow and permeability in the exchange section and non uniform dispersion of molecules in vessels and cannulas The importance of these and other factors for the shape of the outflow curves has been discussed at a recent symposium (Crone and Lassen 1970)

In the majority of our experiments there was a rise in the extraction, followed by a plateau and then a decrease Bassingthwaighte *et al* (1970) have shown by computer modelling that an initial rise in the extraction may be due to differences in longitudinal diffusion rates between the reference and the test substances *In the*

ΔPS ml × min⁻¹ × 100 g⁻¹		ΔPS per cent		Q_{pl} ml × min⁻¹ × 100 g⁻¹	
sucrose	*PEG*	*sucrose*	*PEG*	*sucrose*	*PEG*
0 4±0 3		15±10		9 2±3 6 9 1±3 3 n s	
1 3±0 8	0 3±0 4	39±23	18±13	16 7±2 1 17.5±2 7 n s	14 9±4.5 15 6±5 3 n.s

present experiments this factor does not seem to be of major importance since we have found the same initial increase regardless of whether we used relatively small molecules like sucrose (M W 342) or larger molecules like dextran (M W 17000—19000) (unpublished) Another factor which may be of importance is the existence of differences in the lengths of precapillary vessels and capillaries Variations of this sort will cause differences in transit times which will contribute to an initial rise in the extraction versus time curve In view of the vascular architecture in subcutaneous adipose tissue with a network of capillaries surrounding each adipocyte (Bini 1930 Gersh and Still 1945 Ballard *et al* 1974) it is reasonable to assume that there are appreciable differences in transit times Goresky *et al* (1970) suggested that the situation with varying capillary transit times may exist in the liver and the lung In view of the similarities of the vascular pattern this may also be the case in adipose tissue

The most reliable way of calculating the extraction occurring in all exchange vessels short or long would be to include all values up to the point where back diffusion becomes apparent as indicated by the decline of the extraction—time curve On the basis of these considerations we have used the weighted means of the separate extraction values i e the area method to represent the overall extraction in the vascular bed for each injection

However because of difficulties in evaluating different factors contributing to the shape of the extraction versus time curves other ways of determining the fractional extraction from the present data should be considered Thus if the first part of the curve is disregarded and only the maximal extraction is used (E_{m}) (Bassingthwaighte *et al* 1970) the differences in extraction obtained between control conditions and nerve stimulation will be the same as with the area method although the numerical values of the extraction will be higher the reason being that the extraction versus time curves during control period and nerve stimulation are fairly parallel

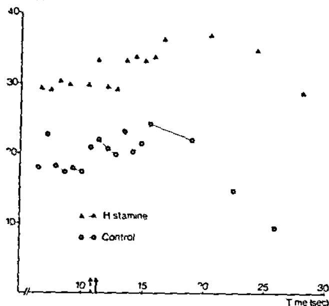


Fig 5 Canine subcutaneous adipose tissue. Extraction of ^{14}C-sucrose during control and during intra arterial infusion of histamine During control papaverine was infused to produce the same blood flow as during infusion of histamine Arrows show time of maximal reference activity Tissue weight = 78 g Plasma flow = 24 ml × min^{-1} × 100 g^{-1} Exp 730475

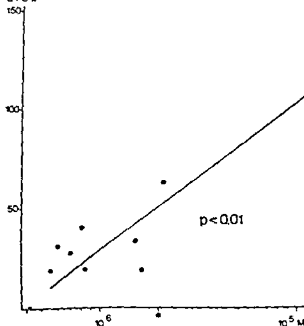


Fig 6 Canine subcutaneous adipose tissue Δ PS for sucrose during i.a. infusion of histamine The concentration indicated on the abscissa is the calculated plasma concentration In order to obtain the same blood flow during control as during histamine infusion prostaglandin E_1 or papaverine were infused during control

We have chosen to express the transcapillary transport in terms of PS products which in theory represent the transcapillary exchange for a given molecule attainable at an infinite blood flow or differently expressed the maximal or limiting clearance for the molecule in the organ in question (Renkin 1959).

In the present experiments the clearance for sucrose under resting conditions was closely related to plasma flow and did not approach a constant level. However when vasodilating agents were administered to produce a maximal blood flow a constant clearance was approached corresponding to a PS product of 3 ml × min^{-1} × 100 g^{-1}. The difference in the clearance values between the undilated and the vasodilated situation is presumably not only due to an elevated total blood flow but is also a consequence of an increased capillary surface area. On the basis of our experiments it is not possible to determine the relative importance of these two factors. However it is reasonable to assume that the increase in surface area produced by the administration of vasodilating agents makes an appreciable contribution to the increase in clearance. This may also be true for those 'resting' experiments in which the plasma flow was above the resting level for adipose tissue (mean plasma flow 7 to 8 ml × min^{-1} × 100 g^{-1}). Thus it seems reasonable to assume that under resting conditions PS for sucrose is slightly below 2 ml × min^{-1} × 100 g^{-1} and the transcapillary transport of sucrose predominantly diffusion limited at plasma flow levels above 8 to 10 ml × min^{-1} × 100 g^{-1}. For PEG the corresponding PS-value appears to be approximately 1 ml × min^{-1} × 100 g^{-1} which is reasonable PEG being a bigger molecule (Alvarez and Yudilevich 1969). From Fig. 3 it is evident that the clearance of PEG approaches a constant level at a plasma flow of about 5 ml × min^{-1} × 100 g^{-1} indicating that at or above this level of plasma flow the transcapillary transport of PEG is mainly diffusion limited. A maximal vasodilatation produced a 100 % increase in PS for PEG. As discussed above for sucrose this increase is presumably due to an enlargement of the capillary surface area.

Even if the clearance is largely independent of blood flow at high flow levels precautions have been taken to keep the blood flow at the same level under control conditions and during stimulation and thus possible influences of blood flow changes on the calculated diffusion capacities have been eliminated. A statistical test showed that the difference in blood flow between the control state and the period of experimental intervention was insignificant ($p > 0.1$) in all groups studied (Table I).

During sympathetic nerve stimulation increases in the PS for sucrose from 3.1 to 4.4 ml × min^{-1} × 100 g^{-1} and for PEG from 2.0 to 2.3 ml × min^{-1} × 100 g^{-1} were found. This corresponds to percental increases of approximately 40 and 20 respectively. Increases in the PS products were seen even at 1 Hz. However there was no statistically significant correlation between the change in the PS product and the stimulation frequency as tested by partial correlation analysis. One reason for this apparent lack of frequency dependency may be that we have relatively few observations above 3 Hz.

The increase in the PS products for sucrose from 3.1 to 4.4 ml × min^{-1} × 100 g^{-1} is evidently not dependent on an increase in resistance since in some experiments the

PS was not determined until the blood flow had spontaneously returned to control levels Furthermore, the increased PS remained elevated for a long period after the end of the stimulation These data uggest that the increa_es in the PS products found in this study are mechanically independent of changes in resistance

The increase in the PS for sucrose and PEG induced by sympathetic nerve ac tivity is presumably a consequence of an increase in the diffusion capacity of the capillary section Such an increase may result from an enlargement of the capillary surface area and/or an increase in permeability In a previous study sympathetic nerve stimulation has been shown to decrease the capillary surface area in sub cutaneous adipose tissue (Linde and Gainer in press) In the present study the capillary surface area has most probably been decreased, especially in the group in which the resistance was increased (Table I) Moreover, the most pronounced in creases in the diffusion capacities were seen during maximal vasodilatation

The increase in the PS product found during sympathetic nerve stimulation may *thus be due to an increased permeability as a consequence of an increased pore size* or an increase in the number of pores per unit surface area From the present data it is not possible to differentiate between these two possibilities However, the fact that the isovolumetric capillary pressure decreases during sympathetic nerve activity supports the suggestion of an increased pore size thus allowing osmotically active substances to penetrate the vascular wall to a larger extent (Rosell *et al* 1974)

It is evident from these experiments that in comparison to other vasodilating histamine has a pronounced effect on PS This additional effect is most probably due to an increased vascular permeability (Spector 1958 Majno and Palade 1961 Haddy *et al* 1972) Thus it is likely that histamine and sympathetic nerve stimulation have similar effects on the permeability in subcutaneous adipose tissue

In summary the present experiments indicate that sympathetic nerve activity and histamine induce an increased vascular permeability for solutes in canine sub cutaneous adipose tissue A permeability change is in agreement with the resul s of other studies in which other parameters such as the capillary filtration coefficient (Öberg and Rosell 1967) and the isovolumetric capillary pressure (Rosell *et al* 1974) have been measured

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References

ALVAREZ O A and D YUDILEVICH Heart capillary permeability to lipid insoluble molecules *J Physiol* (Lond) 1969 202 45—58

BALLARD K T MALMFORS and S ROSELL Adrenergic innervation and vascular patterns in canine adipose tissue *Microvasc Res* 1974

BASSINGTHWAIGHTE J B T J KNOPP and J B HAZELRIG *A concurrent flow model for capillary tissue exchanges* Alfred Benzon Symposium II Munksgaard Copenhagen 1970 60—80

BAI A Vascolorizzione del tessuto dispote e rapporti tra capillarie cellule adipose *Boll Soc ital Biol sper* 1939 *14* 328—369

CHINARD F P G J VOSBURGH and TH EVANS Transcapillary exchange of water and of other substances in certain organs of the dog *Amer J Physiol* 1955 *183* 221—234
CRONE C The permeability of capillaries in various organs as determined by use of the indicator diffusion method *Acta physiol scand* 1963 *58* 292—305
CRONE C and N A LASSEN *Capillary permeability* Alfred Benzon Symposium II Munksgaard Copenhagen 1970
GERSH I and M A STILL Blood vessels in fat tissue Relation to problems of gas exchange *J exp Med* 1945 *81* 219—232
GORESKY C A W H ZIEGLER and G G BACH Capillary exchange modelling *Circulat Res* 1970 *27* 739—764
HADDY F J J B SCOTT and G J GREGA Effects of histamine on lymph protein concentration and flow in the dog forelimb *Amer J Physiol* 1972 *223* 1172—1177
LINDE B and J L GAINER Disappearance of 133Xenon and 125Iodide and Extraction of 86Rubidium in Subcutaneous Adipose Tissue during Sympathetic Nerve Stimulation *Acta physiol scand* 1974 *91* 172—179
MAJNO G and G E PALADE Studies on inflammation I The effect of Histamine and Serotonin on Vascular Permeability An Electron Microscopic Study *J biophys biochem Cytol* 1961 *11* 571
ÖBERG B and S ROSELL Sympathetic control of consecutive vascular sections in canine subcutaneous adipose tissue *Acta physiol scand* 1967 *71* 47—56
RENKIN E Transport of potassium-42 from blood to tissue in isolated mammalian skeletal muscles *Amer J Physiol* 1959 6 1205—1210
ROSELL S Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation *Acta physiol scand* 1966 *67* 343—351
ROSELL S M INTAGLIETTA and G CHISOLM Adrenergic Influence on Isovolumetric Capillary Pressure in Canine Adipose Tissue *Amer J Physiol* 1974 In press
SNEDECOR G W and W G COCHRAN *Statistical methods* The Iowa State University Press Ames Iowa, U S A 1967
SPECTOR W G Substances which affect capillary permeability *Pharmacol Rev* 1958 *10* 475

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From the Section of Pharmacology Division of Experimental Defence Medicine
Research Institute of National Defence Department 1 Sundbyberg Sweden

Choroid Plexus Uptake of Acetylcholine

By

B Winbladh

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Abstract

Winbladh B *Choroid plexus uptake of acetylcholine* Acta physiol scand 1974 *92* 156—164

Acetylcholine (Ach) is accumulated by rabbit choroid plexa *in vitro* by active transport. The transport system seems to be identical to that for choline (Ch) and has a higher capacity Calculated maximal speed varied between 10 and 60 mmol $\times$ min^{-1} $\times$ kg^{-1} wet weight. The choroid plexa contained a significant cholinesterase (ChE) activity which seemed to be localized intravascularly ACh was not metabolized by the tissue *i e* intravascular concentrations of accumulated ACh were low The cholinesterase inhibitor (ChEI) physostigmine inhibited ACh and Ch uptake while the two ChEI s soman and sarin stimulated the uptake of both ACh and Ch. The relative stimulation of the ACh uptake by soman increased with increasing ACh concentration in the incubation medium The stimulation of ACh (10^{-5} M) uptake was not significantly different at soman concentrations ranging from 10^{-3} to 10^{-6} M Soman did not enhance the release of ACh from tissue preincubated at 10^{-4} or 10^{-5} M ACh for 15 min The results are discussed in relation to ACh and Ch transport in brain slices Further possible localizations of the transport mechanism are briefly discussed

Acetylcholine (ACh) is accumulated in certain tissues including brain slices *in vitro* by a saturable process Because of the high cholinesterase (ChE) content of brain slices the studies of ACh uptake in this tissue have mainly been performed in the presence of cholinesterase inhibitors (ChEI s) in order to prevent ACh breakdown However the uptake is competitively inhibited by the ChEI physostigmine *but not* by the other ChEI s soman and tabun (Schuberth and Sundwall 1967 Polak 1969) Further these workers showed that the uptake is competitively inhibited by several drugs including both cholinergic agonists and antagonists In addition choline (*Ch*) is taken up by brain slices (Schuberth *et al* 1966) and decreases the uptake *rate* of ACh (Polak 1969) However Schuberth nad Sundwall (1967) pointed out *some* important differences in the influence of the above mentioned drugs on the uptake of ACh and Ch respectively which speaks in favour of at least partly *different* uptake mechanisms for these two substances in brain slices

Choroid plexa accumulate Ch by active transport. This transport mechanism is shared with a number of other quaternary and tertiary amines (Erikson and Winbladh 1971)

Histologically the choroid plexus consists of a specific ependyma, connective tissue, a fenestrated endothelium and blood. The nervous system is rather sparse (Maxwell and Pease, 1956). Thus the composition of this tissue is much less heterogeneous than that of the brain.

Preliminary investigations on rabbit choroid plexa showed a low but significant content of ChE activity. On the other hand, histochemical investigations have not revealed any ChE in the choroid plexus tissue itself (Manocha and Shanta, 1970). Thus it appeared as all ChE activity might be located intravascularly. If this was the case, analysis of the content of Ch in choroid plexus after incubation with ACh would give an idea of the compartmentation of accumulated ACh.

From the above discussed findings the following four questions came up:

1. Is ACh actively accumulated by choroid plexus in spite of its probable lack of cholinergic structures?
2. Are there completely or partially different uptake systems for choline and acetylcholine?
3. Do ChEI's as physostigmine, sarin and soman influence the transport in spite of the low concentrations of ChE in the choroid plexus?
4. To what extent is the accumulated ACh localized intravascularly, *i.e.* can the transport mechanism be localized?

Methods

Adult Small chinchilla rabbits were killed by air embolus and lateral ventricle choroid plexa were rapidly dissected out. The tissue was divided into two pieces, rinsed in ice cold Krebs-Henseleit buffer and preincubated 10 min at 37 °C in 2 ml of the same buffer in a Warburg apparatus under gentle shaking and gassing with 93.5 % O_2 and 6.5 % CO_2. After this time labelled ACh, carrier ACh and other drugs were added from the side arm of the incubation bottle. Standard incubation time was 15 min. After the incubation the plexa were rinsed in ice cold buffer without ACh and excess buffer was removed with a filter paper. Tissue and incubation medium content of radioactivity was determined by liquid scintillation counting, the former after oxygen combustion as described elsewhere (Eriksson and Winbladh, 1971). In some experiments the identity of the radioactivity was investigated by high voltage electrophoresis (pyridine acetate buffer 0.2 M, pH 4.6, Whatman no. 1, 50 V/cm, 2 h) of acid ethanol extracts of tissue homogenates as described elsewhere (Eriksson and Winbladh, 1971). Carrier Ch and ACh were added to the extracts and the corresponding spots on the electropherograms were localized with iodine vapour. The radioactivity content of the spots was determined by water elution or oxygen combustion and liquid scintillation counting. Mean recovery for ACh was 6[illegible] ± 5 (n = 3) per cent of spotted amount. The estimation of the kinetic parameters for the uptake corresponding to V_{max} and K_m in the Michaelis-Menten equation was done by an iterated hyperbolic fitting in the velocity versus concentration plot performed with a computer as described elsewhere (Eriksson and Winbladh, 1971). At high tissue medium ratios (T/M) there is a significant reduction of incubation medium concentration during the incubation. This has been compensated for by excluding the lowest medium ACh concentrations where T/M > 100 from the calculations of the parameters and by using mean incubation concentration = (initial + final concentration)/2 instead of initial concentration in the calculations, as suggested by Lee and Wilson (1971).

In a separate series of experiments the ChE activity of choroid plexus homogenate, heart and ear vein blood was determined by the method of Ellman *et al.* (1961). In some of these experiments ^{125}I albumin was injected into an ear vein 5 min before killing the animal. The radioactivity in the choroid plexus and whole blood from the heart was then analyzed in a scintillation detector (Picker Autowell II). The same type of experiment was also performed on rabbits where the carotids postmortally had been washed with isotonic saline until the brain and choroid plexa were without macroscopically visible red vessels.

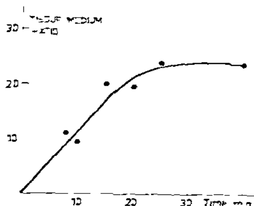


Fig. 1. Uptake of acetylcholine (ACh) in choroid plexus with time. Each point represents one experiment.

Drugs used were (³H acetyl)-choline iodine (NEN, Frankfurt, Germany, 6° Ci/mol.) ¹²⁵I albumin (CNTS Paris, France ~0 mg/ml 0.010 mCi/ml) acetylcholine iodide (East man, N.Y., USA), physostigmine salicylate (Military Pharmacy, Stockholm, Sweden), physostigmine sulphate and acetylthiocholine iodide (Sigma, St. Louis, USA), soman and sarin (synthesized by the Chemical Division, Res. Inst. Natl. Def., Sweden.

Results

Uptake of ACh. Choroid plexus accumulated ACh against a concentration gradient. The uptake increased approximately linearly with time during the first 15—20 min of incubation (Fig. 1). Further, the uptake showed saturation characteristics (Fig. 2) and was highly reduced by incubation at 0 °C (98 % reduction at 2—10⁻⁵ M ACh) and nitrogen gassing (99 % reduction at 10⁻⁵ M ACh). However, the rate of accumulation varied considerably between different animals and experiments. Two extreme curves each representing one experiment are shown in Fig. 2 (inset). A large individual variation in the choroid plexus accumulation rate of other drugs has been found in earlier experiments (Eriksson and Winbladh 1971, Winbladh 1972, Welch 1962, Tochino and Schanker 1965 *a*). Our material does not permit any conclusions whether the interindividual variations were smaller within a certain litter. The calculated uptake parameters for the experiments in Fig. 2 are given in Table I. It is seen that both V_{max} and K_m vary between these three experiments. Consequently, the differences in transport capacity might depend on differences in carrier characteristics as well as in carrier abundance.

After 15 and 75 min incubation all radioactivity on the electropherograms of tissue homogenate and incubation medium was recovered from the acetylcholine spot. No significant amounts were found as choline or phosphorylcholine.

Influence of Ch on ACh uptake. Incubation with choline 10⁻⁴ M gave an increasing inhibition of ACh uptake from almost 0 % at 10⁻⁵ M to 75 % at 10⁻⁵ M ACh concentration. The double reciprocal plots suggested a competitive inhibition as well as the calculated uptake parameters, which are given in Table I. Incubation of ACh 10⁻⁵ M with Ch concentrations increasing from 10⁻⁴ to 5 · 10⁻³ M

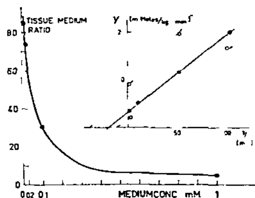


Fig 2

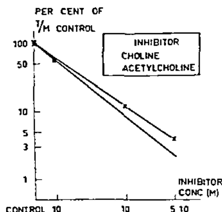


Fig 3

Fig 2 Influence of incubation medium concentration of acetylcholine (ACh) on tissue medium ratio of ACh Mean from two experiments *Inset* Double reciprocal plots of same experiment as in tissue medium ratio curve (solid line) and two separate experiments with extremely high and low uptake rates (dotted lines) v = velocity s = medium conc

Fig 3 Influence of different medium acetylcholine (ACh) and choline (Ch) concentrations on tissue medium ratio for Ch and ACh (10^{-5} M) respectively Each point represents mean of 2 expts

gave an increasing inhibition of the ACh uptake (Fig 3) up to 97 % at the highest Ch concentration Similarly incubation of Ch 10 M with ACh 10^{-3} and 5 10^{-3} M gave respectively 88 and 96 per cent reduction of the Ca uptake (Fig 3)

Influence of ChEI on ACh uptake Physostigmine salicylate 10^{-4} M gave an almost complete inhibition of the uptake of ACh Since the T/M at 10^{-4} 2 10 and 10^{-5} M ACh concentration did not significantly exceed that of 10^{-3} namely 0 6—0 8 it is not possible to say if the inhibition is competitive or not Physostigmine sulphate (10^{-4} M) likewise gave a pronounced inhibition (88 and 94 %) of ACh

Table I Choroid plexus uptake characteristics* for acetylcholine (ACh)

$V_{max} \pm$ S E mmol min × kg	$K_m \pm$ S E mM	Number of animals Number of tissue samples
0 32 ± 0 01	0 057 ± 0 006	6/24
0 12 ± 0 01	0 073 ± 0 005	1/4
0 54 ± 0 01	0 078 ± 0 007	1/4
0 43 ± 0 03	0 036 ± 0 011	1 5 6
In presence of choline 0 1 mM		
0 60 ± 0 11	0 25 ± 0 17	1 5/6 *

Parameters as in the Michaelis-Menten equation

Paired experiment on 3 rabbits with choroid plexa divided into 2 parts one incubated with one without choline Calculated

$$K_i = \frac{0\ 1}{0\ 25\ 0\ 035 - 1} \approx 0\ 0?\ \text{mM}$$

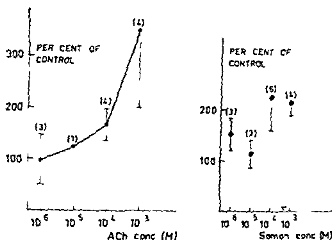


Fig 4 *Left* Effects of different acetylcholine (ACh) concentrations on soman (10^{-4} M) stimulation of ACh uptake (*n*) = number of experiments Mean ± S E *Right* Effects of different soman concentrations on stimulation of ACh (10^{-4} M) uptake Mean ± S E

($2 \cdot 10^{-5}$ M) uptake In addition the uptake of choline ($2 \cdot 10^{-5}$) was inhibited by physostigmine salicylate (10^{-4} M) by 94 and 89 per cent in 2 expts respectively

In contrast to physostigmine both sarin and soman (10^{-4} M) increased the T/M of ACh (10^{-4} M) in comparison with the other half of the plexus serving as control (Table II) The stimulation varied in different experiments and with both drugs there was a tendency to higher stimulation the lower the T/M of the control (Table II) A similar stimulation of Ch uptake was found with soman (Table II)

The relative stimulation of ACh uptake by soman seemed to increase with increasing ACh concentration in the incubation medium Because of the rather large interindividual variations in uptake rate mentioned above these experiments had to be performed with one half of each plexus serving as control and with the two plexa from one animal at different ACh concentrations 6 such experiments were performed and all showed an increasing relative stimulation with increasing ACh concentration Fig 4 (left) shows the mean values of these experiments When the

TABLE II Effects of soman and sarin on acetylcholine (ACh) and choline (Ch) uptake in choroid plexa

	Soman 10^{-4} M		Sarin 10^{-4} M	
	T M drug / T M control	% of control	T M drug / T/M control	% of control
ACh 10^{-4} M	62 27	230		
	1 6 32	550	27 11	257
	128 96	136	85 15	560
			146 87	167
Ch 10^{-4} M	109 73	148		
	227 75	307		
	109 83	131		
	148 155	94		

TABLE III Effect of soman ~⁴ M on release of acetylcholine (ACh) from preincubated choroid plexa

Preincubation		Reincubation		
ACh conc M	tissue medium ratio *	tissue medium ratio ± S D		number of pairs
		soman	control	
10	15	313±14	374±73	2
10	170	418±45	435±51	6

* One control and one soman treated from each di ıded preincubated plexus
* Calculated from total dpm/mg plexus in reincubation medium divided by dpm/μl in preincubation medium

soman concentration was varied from 10^{3} to 10^{6} M at an ACh concentration of 10^{5} M no conclusive changes in stimulation were found in the 5 expts performed (Fig 4 right) Soman did not stimulate uptake of ACh above controls when incubations was performed in nitrogen atmosphere This shows that the stimulated uptake is energy dependant Further soman (10^{4} M) did not influence choroid plexus oxygen uptake as measured with the standard Warburg technique in 3 expts

Influence of ChEI on the release of ACh from choroid plexa The finding of a stimulation of ACh and Ch uptake by soman and sarin in contrast to physostigmine which inhibited the uptake seemed remarkable A possible explanation would be that ACh and Ch also possessed a soman and sarin but not physostigmine sensitive transport system working in the opposite direction *i e* releasing ACh and Ch from the tissue This hypothesis was investigated in the following way The choroid plexa *were preincubated 15 min in labelled ACh 10^{5} or 10^{3}* M then rinsed and reincubated in isotope free buffer with ACh 10^{3} M in order to block reuptake of released labelled drug The reincubation was done without (control) and with soman (10^{4} M) Reincubation T/M s of radioactivity were then compared between the soman and the control group As seen in Table III no significant differences were found between soman treated and control tissues T/M s after the first incubation were calculated from the sum of total radioactivity in tissue and incubation medium *in the second incubation divided* by the plexus weight and concentration of radio activtiy in the first incubation medium

ChE and ¹²⁵I-albumin content of charoid plexa After killing the rabbits with an air embolus the choroid plexa contained visible blood filled vessels and the homogenates possessed a significant ChE and ¹ ⁵I activity (Table IV) Histologic examination* revealed that even the capillaries contained a high number of erythrocytes often congested in lacunae The intravascular volume calculated on the basis of the ¹ ⁵I *content of the tissue as compared to heart blood* was around 15—20 per cent (Table IV) The ChE content of the choroid plexa was considerably

* Paraff n embedding hemato ylin-eosin stain

TABLE IV Choroid plexus content of ¹²⁵I and cholinesterase activity (ChE)*

	ChE activity		¹²⁵I	
	Choroid plexus (Mean ± S E)	Blood choroid plexus Mean ± S E	of plasma activity Mean ± S E	n
Non flushed tissue	665 ± 110	900 ± 35	17 ± 2	
Flushed tissue	(120 ± 30)	875 ± 55	7 ± 1	

* μ mole acetylthiocholine hydrolyzed per min and g tissue
Values within brackets less than 1 3 times spontaneous hydrolysis n = number of experiments

higher than the ¹²⁵I content in relation to plasma (Table IV) However after flushing of the carotid arteries with saline until no red coloured vessels were visible in the choroid plexa the plexus content of ChE fell to values not significantly higher than the background (Table IV) Thus almost all of the ChE activity seems to be localized intravascularly The reason for the high content of ChE in relation to the ¹²⁵I content in the non flushed tissue might be a trapping of large amounts of erythrocytes in the tissue as compared to the amount of plasma possibly due to the method of sacrifice This view is supported by the finding of a congested appearence of the capillaries in the histological examination Because of colour interference the exact proportion of ChE in plasma and red blood cells could not be determined with the colorimetric method. However as determined with an electrometric method (Tammelin 1953) with ACh as substrate (0 08 M pH 8 2) the ChE activity per ml red blood cells was about half of that per ml of plasma

Discussion

ACh is accumulated by choroid plexa in vitro by a mechanism which fulfills the conventional criteria for active transport i e uptake against a concentation gradient by an energy dependant saturable mechanism The transport system is shared with choline Since high concentrations of both dugs inhibited the uptake of the other by more than 95 % there appears to be identical uptake systems for the two drugs in this concentration range This conclusion is of course valid only if incubation medium concentrations of ACh and Ch in mM range do not influence the tissue in any other way The finding of linear uptake curves in the double reciprocal plots suggests that there is no significant concentration dependant influence on the tissue besides that on the uptake The choroid plexus uptake of ACh as well as Ch is inhibited by physostigmine which is a secondary amine It has been shown that primary and tertiary amines are competitive inhibitors of the uptake of some quaternary amines in rabbit choroid plexus (Tochino and Schanker 1966 Eriksson and Winbladh 1971 It thus seems possible that the effect of physostigmine on the uptake is a competitive inhibition although this was not actually shown In brain slices physostigmine is a competitive inhibitor of ACh uptake (Schuberth

and Sundwall 1967, Polak 1969) but does not affect the uptake of choline significantly (Schuberth *et al.* 1966). The uptake of choline is not due to a tissue metabolism either in brain slices or choroid plexus since almost 80 (Schuberth *et al.* 1966) and 100 (Eriksson and Winbladh 1971) per cent of the tissue radioactivity could be recovered as unchanged choline. Consequently there seems to be a qualitative difference in bulk ACh and Ch transport in mouse brain slices and rabbit choroid plexus at medium concentrations ranging from 5 to 0.01 mM. During the preparation of this manuscript several studies on choline uptake in rat brain synaptosomes has revealed 2 uptake systems for choline: one high affinity system ($K_m \approx 10^{-6}$ M) associated with ACh synthesis and one low affinity system ($K_m \approx 10^{-4}$ M) not associated with ACh synthesis (Yamamura and Snyder 1972, Haga and Noda 1973, Kuhar *et al.* 1973).

The mechanism behind the stimulation of the uptake found with sarin and soman is not evident. Soman did not influence the uptake of ACh in brain slices (Polak 1969) but a weak stimulation of choline uptake was found with sarin (30 %, Schuberth *et al.* 1966). In the present experiments the relative influence of the two ChEI's seemed to increase with increasing ACh concentration in the medium. Neither sarin nor soman had any influence on the release of ACh from preincubated slices suggesting that the mechanism behind the stimulation is not a block of an ACh releasing mechanism. The oxygen consumption of the choroid plexa was not influenced by soman (10^{-4} M) in preliminary experiments. At present no definite explanation for this stimulation of ACh and Ch uptake can be offered. Anyhow it can be concluded from these experiments with ChEI's that their effects do not seem to be related to their influence on ChE.

The choroid plexa in the present investigation normally contained a significant amount of ChE activity. The almost complete disappearance of this activity after flushing with saline makes it likely that the ChE activity was localized intravascularly. However a large part of the ChE activity might have been within the erythrocytes and possibly not accessible for the ACh. The probable plasma ChE activity in the choroid plexus can be calculated with the figures for their ^{131}I content and the heart plasma ChE activity. This calculated activity is in the order of 0.1 μmol ACh per minute and gram plexus, i.e. 1.5 and 7.5 μmoles in the used incubation times of 15 and 75 min respectively. Choroid plexus concentration of ACh was around 5 μmol/g tissue in the experiments where search for metabolites was done. The ChE activity in the capillaries would consequently be sufficient to split a significant part of the ACh in the plexus. Since no degradation of ACh was found the intravascular concentration of ACh must have been very low.

No quantative evaluation has been reported for the relative volumes of the different constituents of rabbit choroid plexa. In pig choroid plexa this has been done by a mechanical separation of epithelium and stroma (Friedenwald, Herrmann and Buka 1942). The epithelium constituted one third, blood one third, stroma and walls of blood vessels one sixht each of the dry weight. If the ACh is mainly accumulated in the capillary walls one would expect that the diffusion into the capil

lary lumen would be sufficient for a detectable hydrolysis of ACh. Thus it appears most likely that the drug is accumulated in the epithelium i.e the transport mechanism is located in the free ependymal border. This view was also put forward by Cohen (1969) who found an active uptake of iodide in intact choroid plexa *in vivo* and in addition in isolated ependymal cells from choroid plexa.

In conclusion rabbit choroid plexa *in vitro* accumulate ACh at a high rate which is in the same order of magnitude as that for Ch. ACh and Ch seem to share the same transport system which is independant of the ChE in the tissue. The ChE is mainly localized intravascularly in contrast to the ACh. The ChEI s soman and sarin stimulated the uptake of ACh and Ch in contrast to physostigmine, which inhibited the uptake.

The author is indebted to Mr S. Zillén for skillful technical assistance and to Dr G. Heimbürger for the electrometric determination of ChE activity.

References

COHEN L. A. Uptake of iodide by choroid plexus in vitro and location of the iodide pump. *Amer J Physiol* 1969 217 89—97

ELLMAN G L. K. D COURTNEY V. ANDRES JR and R. M FEATHERSTONE A new and rapid colorimetric determination of acetylcholine esterase. *Biochem Pharmacol* 1961 7 88—95

ERIKSSON K.-H. and B WINBLADH Choroid plexus uptake of atropine and methylatropine *in vitro*. *Acta physiol scand* 1971 83 300—302

FRIEDENWALD J S. H. HERRMAN and R. BUKA, The distribution of certain oxidative enzymes in the choroid plexus. *Bull Johns Hopkins Hosp* 1942 70 1—13

HARA, T and H. NODA Choline uptake systems of rat brain synaptosomes. *Biochim biophys Acta (Amst)* 1973 291 564—575

KUHAR M J V. H. SETHY R. H. ROTH and G K. AGHAJANIAN Choline Selective accumulation by central cholinergic neurons. *J Neurochem* 1973 20 581—593

LEE, H J and J B WILSON Enzymic parameters Determination of V and Km. *Biochim biophys Acta (Amst)* 1971 242 519—522

MANOCHA S L. and T R. SHANTA, *Macaca mulatta Enzyme histochemistry of the nervous system* Academic Press New York 1970

MAXWELL D S and D C PEASE The electron microscopy of the choroid plexus. *J biophys biochem Cytol* 1956 2 467—474

POLAK R L. The influence of drugs on the uptake of acetylcholine by slices of rat cerebral cortex. *Brit J Pharmacol* 1969 36 144—152

SCHUBERTH J and A SUNDWALL Effects of some drugs on the uptake of acetylcholine in cortex slices of mouse brain. *J Neurochem* 1967 14 [illegible]—812

SCHUBERTH J A. SUNDWALL B SÖRBO and J-O LINDELL Uptake of choline by mouse brain slices. *J Neurochem* 1966 13 347—352

TAMMELIN L. E An electrometric method for the determination of cholinesterase activity. *Scand J clin Lab Invest* 1953 5 267—270

TOCHINO Y and L S SCHANKER Active transport of quaternary ammonium compounds by the choroid plexus in vitro. *Amer J Physiol* 1965a 208 [illegible]

TOCHINO Y and L S SCHANKER Transport of serotonin and norepinephrine by the rabbit choroid plexus in vitro. *Biochem Pharmacol* 1965b 14 1557—1566

WELCH K. Active transport of iodide by choroid plexus of the rabbit in vitro. *Amer J Physiol* 1962 202 [illegible]—760

YAMAMURA A I and S H SNYDER Choline high-affinity uptake by rat brain synaptosomes. *Science* [illegible]

Acta physiol scand 1974 92 165—174

From the Department of Physiology and Biophysics University of Lund Sweden

Role of Tissue Hyperosmolality in Functional Vasodilatation in the Submandibular Gland

By

J Lundvall and J Holmberg

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Abstract

Lundvall J and J Holmberg *Role of tissue hyperosmolality in functional vasodilatation in the submandibular gland* Acta physiol scand 1974 *92* 165—174

Parasympathetic activation of the submandibular gland in the cat was found to cause considerable regional tissue hyperosmolality concomitant with the functional hyperemia response The hyperosmolality as traced in the venous effluent, was present at all rates of chorda nerve stimulation (0 25—20 Hz) Osmolality was clearly raised already during the initial phase of the dilator response and reached plateau values during the later steady state hyperemia response In the latter phase it could exceed the resting control level by as much as 50 mOsm/kg H_2O There was a direct relation between the degree of venous hyperosmolality and the dilator response during low and moderate rates of chorda stimulation Experimental hyperosmolality in the resting gland produced by intra arterial infusion of hypertonic solutions evoked a pattern of dilator response resembling that observed during chorda stimulation Graded experimental hyperosmolality of magnitudes similar to those seen during chorda stimulation evoked progressively increasing dilatations of the resistance vessels Comparison of the dilator effects during chorda stimulation and during hypertonic infusion at comparable levels of venous hyperosmolality indicated that tissue hyperosmolality contributed significantly to both the initial and maintained functional hyperemia response in the gland The factor seemed to be of special importance at low and moderate rates of chorda excitation This conclusion was supported by a separate series of experiments in which the effects of atropine on the functional hyperemia response and on the hyperosmolality were studied

The cause of functional vasodilatation in glandular tissue mainly studied in salivary glands is still under debate The functional hyperemia in salivary glands has been attributed to two different mechanisms Thus Hilton and collaborators (*e g* Hilton and Lewis 1955 and 1956 Hilton and Torres 1970 Gautvik Hilton and Torres 1970) have presented strong evidence for the hypothesis that the vasodilatation is induced by a bradykinin like substance released by the proteolytic enzyme kallikrein from an *a* globuline substrate in plasma Schachter and colleagues (*e g* Bhoola *et al* 1965 Morley Schachter and Smaje 1966 Schachter and Beilenson 1968 Karpinski Barton and Schachter 1971) on the other hand reached the conclusion that the vasodilatation can be entirely attributed to cholinergic vasodilator fibre activation an opinion already expressed in 1872 by Heidenhein In recent studies by

Gautvik (1970a, b and c) it was claimed that both these mechanisms contribute in an important way to the hyperemia response, the vasodilatation being initiated mainly by acetylcholine and then maintained by the kallikrein bradykinin mechanism.

During secretion the metabolic activity of the salivary glands is greatly increased (for ref. see Terroux, Sekelj and Burgen 1959). The question may therefore be raised if, besides the mentioned specific dilator mechanisms, more "conventional" metabolic dilator factors could be causally linked to the hyperemia response. In exercising skeletal muscle the markedly increased metabolism leads to a pronounced regional hyperosmolality, which has been shown to be an important causal factor in the exercise hyperemia response (Mellander *et al.* 1967, Lundvall 1972). The present study shows that a pronounced tissue hyperosmolality develops in the cat's submandibular gland during parasympathetic activation and suggests that this factor contributes to the functional vasodilator response in this tissue as well.

Methods

19 cats of both sexes, weighing 2.5—4.8 kg, were used. The animals were anesthetized with chloralose (50 mg/kg b.w.) and urethane (100 mg/kg b.w.) after induction with ether. Body temperature was maintained at 38±0.5 C. The submandibular gland was exposed unilaterally in the neck and all connections between the glandular tissue and the surrounding structures were divided with the exception for the parasympathetic nerves, the glandular duct, the submandibular artery and the main vein emerging from the gland. The salivary duct was cannulated with a polyethylene catheter of largest possible bore. After heparinization (3—5 mg/kg b.w.) the external jugular vein was cannulated and all tributaries except that from the submandibular gland were ligated. Venous outflow from the gland was diverted through an optical drop-recorder unit for continuous registration of blood flow and then returned to the animal via a funnel connected to the right femoral vein. Venous outflow pressure was adjusted to heart level. In some of the experiments an arterial shunt circuit, used for close arterial infusions to the gland, was inserted between the brachial artery and the common carotid artery. All branches of the common carotid artery except the submandibular were then ligated. The complete cessation of the blood flow from the gland upon clamping the arterial shunt indicated that no collaterals remained, which was considered important in order to obtain thorough mixing of infused solutions with blood (see below). Arterial inflow pressure was monitored from the right femoral artery or from a T tube in the arterial shunt when the latter was used.

The distal end of the severed chorda lingual nerve was stimulated with a bipolar ring electrode using supramaximal square wave shocks (6—8 V, 2 ms) at frequencies of 0.5 to 20 Hz.

Isotonic and hypertonic (600—1200 mOsm/kg H_2O) glucose or xylose solutions were infused into the gland via the arterial shunt at rates of 0.05—0.30 ml/min using a constant infusion apparatus. Attention was paid to the problem of thorough mixing with blood to obtain an even distribution of the infused solutions in the gland (*cf.* Lundvall 1972). Mixing was achieved by a vibrator device attached to the arterial shunt tubing and was found effective in pilot dye infusion tests. Such vibration did not cause liberation of vasoactive agents in the blood or blood cell aggregation as evidenced by special test experiments (*cf.* Lundvall 1972).

Venous samples were collected at intervals for determination of plasma osmolality. The "dead space" volume of the outflow tubing was discarded during sampling. Plasma osmolality was determined by thermistor cryoscopy (Osmometer 31 LAS, Advanced Instruments, Inc.). Each sample was measured twice. When different readings were obtained the mean value was used.

Arterial pressure, glandular blood flow and submandibular secretion (the latter registered as drops of known volume) were recorded on a Grass polygraph.

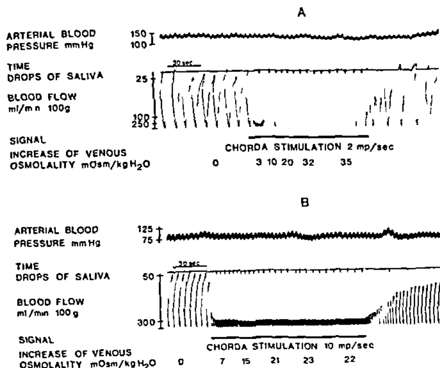


Fig 1 Typical changes of blood flow and regional venous osmolality in the cat submandibular gland during low (2 Hz) and intense (10 Hz) chorda nerve fibre activation The induced saliva secretion (each drop 0 07 ml) is also shown.

Results

Chorda stimulation experiments

Fig 1 illustrates typical changes in blood flow and in venous plasma osmolality observed in the submandibular gland at low (panel A) and high (panel B) rates of chorda stimulation The evoked saliva secretion is also shown (drop volume 0 07 ml) In the control period before stimulation at 2 Hz blood flow was about 25 ml/min× 100 g tissue and venous osmolality was 318 mOsm/kg H O It can be seen that blood flow started to increase very shortly after the commencement of chorda stimulation to reach a maximal value of about 270 ml/min×100 g within some 10 s (initial dilator response) After this response blood flow decreased markedly and attained a steady state hyperemia level of about 100 ml/min×100 g maintained during the remainder of the stimulation period (below denoted maintained dilator response) This flow pattern was always observed at low stimulation rates (0 25—2 Hz) Venous osmolality rose quickly upon commencement of chorda stimulation to reach a maximal and maintained level of 32—35 mOsm above the control level after about one min During high frequency stimulation (10 Hz panel B) blood flow increased rapidly and attained within some 30 s an approximate steady state level

Gautvik (1970 a b and c) it was claimed that both these mechanisms contribute in an important way to the hyperemia response the vasodilatation being initiated mainly by acetylcholine and then maintained by the kallikrein bradykinin mechanism

During secretion, the metabolic activity of the salivary glands is greatly increased (for ref see Terroux Sekelj and Burgen 1959) The question may therefore be raised if besides the mentioned specific dilator mechanisms more conventional metabolic dilator factors could be causally linked to the hyperemia response In exercising skeletal muscle the markedly increased metabolism leads to a pronounced regional hyperosmolality which has been shown to be an important causal factor in the exercise hyperemia response (Mellander *et al* 1967 Lundvall 1972) The present study shows that a pronounced tissue hyperosmolality develops in the cats submandibular gland during parasympathetic activation and suggests that this factor contributes to the functional vasodilator response in this tissue as well

Methods

19 cats of both sexes weighing 2 5—4 8 kg were used The animals were anesthetized with chloralose (50 mg/kg b w) and urethane (100 mg/kg b w) after induction with ether Body temperature was maintained at 38±0 5 C The submandibular gland was exposed unilaterally in the neck and all connections between the glandular tissue and the surrounding structures were divided with the exception for the parasympathetic nerves the glandular duct the submandibular artery and the main vein emerging from the gland The salivary duct was cannulated with a polyethylene catheter of largest possible bore After heparinization (3—5 mg/kg b w) the external jugular vein was cannulated and all tributaries except that from the submandibular gland were ligated Venous outflow from the gland was diverted through an optical drop recorder unit for continuous registration of blood flow and then returned to the animal via a funnel connected to the right femoral vein Venous outflow pressure was adjusted to heart level In some of the experiments an arterial shunt circuit used for close arterial infusions to the gland was inserted between the brachial artery and the common carotid artery All branches of the common carotid artery except the submandibular were then ligated The complete cessation of the blood flow from the gland upon clamping the arterial shunt indicated that no collaterals remained which was considered important in order to obtain thorough mixing of infused solutions with blood (see below) Arterial inflow pressure was monitored from the right femoral artery or from a T tube in the arterial shunt when the latter was used

The distal end of the severed chorda lingual nerve was stimulated with a bipolar ring electrode using supramaximal square wave shocks (6—8 V 2 ms) at frequencies of 0 75 to 20 Hz

Isotonic and hypertonic (600—1200 mOsm/kg H_2O) glucose or xylose solutions were infused into the gland via the arterial shunt at rates of 0 05—0 30 ml/min using a constant infusion apparatus Attention was paid to the problem of thorough mixing with blood to obtain an even distribution of the infused solutions in the gland (*cf* Lundvall 1972) Mixing was achieved by a vibrator device attached to the arterial shunt tubing and was found effective in pilot dye infusion tests Such vibration did not cause liberation of vasoactive agents in the blood or blood cell aggregation as evidenced by special test experiments (*cf* Lundvall 1972)

Venous samples were collected at intervals for determination of plasma osmolality The dead space volume of the outflow tubing was discarded during sampling Plasma osmolality was determined by thermistor cryoscopy (Osmometer 31 LAS Advanced Instruments Inc) Each sample was measured twice When different readings were obtained the mean value was used

Arterial pressure glandular blood flow and submandibular secretion (the latter registered as drops of known volume) were recorded on a Grass polygraph

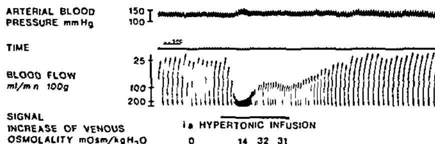


Fig 3 Typical pattern of blood flow changes produced in the resting submandibular gland by i a. hypertonic infusion The observed increases of venous osmolality above control level during the peak flow response and during the steady state flow response are shown

stimulation period even if prolonged (tested up to 8 min) The extent of venous hyperosmolality in the initial (10—15 s) phase of chorda stimulation was in most animals directly related to the stimulation frequency The degree of hyperosmolality in the venous effluent when fully developed similarly increased with increasing stimulation rates up to 4 Hz at which frequency the hyperosmolality was usually most pronounced and could amount to 50 mOsm/kg H O At higher stimulation rates as mentioned the increa e of venous osmolality was less pronounced but still far above the control level (*cf* Fig 2) Yet this decline of venous osmolality at high stimulation rates might not necessarily reflect a corresponding decrease of extravascular hyperosmolality but could be caused by a gradually impaired transcapillary osmolar extraction as blood flow and therefore probably also capillary flow velocity increases

Fig 2 summarizes the results obtained during chorda stimulation (open and closed circles) and shows the evoked dilator effects of the resistance vessels plotted versus the concomitantly observed venous hyperosmolality The data are classed with regard to rate of chorda stimulation (see legend) and given as mean values ± S E The open circles depict effects observed 10—15 s after the commencement of the stimulation (in which phase the initial dilator re ponse at low stimulation rates was evoked) and the closed circles the steady state effects observed 1—2 min after the onset of stimulation The dilator effects of chorda stimulation are expressed in per cent of the maximal dilator response evoked by a supramaximal dose of papaverine given intra arterially in repetitive tests to each animal This mode of expressing the hyperemia response was considered more reliable than mere calculation from the vascular conductance value in the control period (*cf* Myers and Honig 1969 Lundvall 1972) This because the calculated dilator effects would otherwise be much influenced by a varying control vascular conductance and by a varying maximal vascular conductance in different animals Since in the present series of experiments mean control blood flow was 37 ± 2 (S E) and mean blood flow at maximal vasodilatation 528 ± 34 ml/min × 100 g at a perfusion gradient of 100 mm

Hg it should be noted that each 10 per cent change along the ordinate (Fig 2) corresponds to quite a significant blood flow increase or on the average, 49 ml/min × 100 g tissue

The magnitude of the initial dilator response to chorda stimulation (Fig 2, curve A) showed a direct relation to the degree of venous hyperosmolality up to a stimulation frequency of 6—10 Hz, where vascular conductance averaged 58 per cent of that during maximal vasodilatation and the evoked venous hyperosmolality was 10 mOsm excitation at 20 Hz caused no further increase of the dilator response and the venous hyperosmolality was somewhat less pronounced The maintained dilator response (curve B) increased with increasing venous hyperosmolality in the stimulation frequency range up to 4 Hz at which rate vascular conductance was 31 per cent of that at maximal dilatation and the evoked venous hyperosmolality was 29 mOsm Higher stimulation frequencies caused further and considerable increases of vascular conductance, at 20 Hz the dilatation was 83 per cent of the maximum evoked by papaverine The evoked hyperosmolality in the venous effluent however, was less pronounced than at 4 Hz Note that blood flow was measured *on the venous side The fact that plasma fluid is lost by transcapillary exchange* and transformed into saliva during chorda stimulation may therefore explain that a maximal blood flow increase was not observed during intense activation of the gland

Hypertonic infusion experiments

The fact that chorda excitation besides the dilator response of the glandular resistance vessels also evokes venous (tissue) hyperosmolality could indicate a causal relation between the two phenomena To test this hypothesis the effect of hyperosmolality *per se* on the resistance function was analysed by close arterial infusion of hypertonic solutions of glucose or xylose to the resting gland To be of relevance for the present problem the infusion rates were adjusted so as to create osmolar changes in the resting gland (venous blood) resembling those encountered in the active gland both with regard to magnitude and time course Fig 3 illustrates the most commonly observed pattern of blood flow response during such experimental hyperosmolality in this case elicited by xylose infusion Blood flow was about 25 ml/min × 100 g in the control period and increased rapidly in response to the hypertonic infusion to reach an initial peak value of about 190 ml/min × 100 g within some 20 s It then decreased and an approximate steady state hyperemia level of 80—100 ml/min × 100 g was obtained some 60 s after the commencement of the flow increase (steady state response) Venous osmolality increased by 14 mOsm during the peak response and by about 30 mOsm during the steady state response Upon cessation of the infusion there was a gradual recovery of vascular tone The dilator effects almost entirely could be ascribed to the induced hyperosmolality since an isotonic control infusion (not shown in the figure) had little influence on flow

The dilator effects in the resistance vessels to graded experimental hyperosmolality during the peak response and the early steady state response were analysed in 8

animals The data are plotted in Fig 2 versus the concomitantly observed increase of venous osmolality up to 40 mOsm above the control value before infusion (open squares peak response closed squares steady state response) The diagram shows classed mean values ($\pm$S E) for 10 mOsm ranges and refers to both glucose and xylose infusions (no difference in the dilator effect between the two substances was found) Flow effects if present of isotonic control infusions were compensated for in the diagrams

It can be seen that experimental hyperosmolality can evoke considerable dilatation of the resistance vessels and that both the peak and the steady state response increased in relation to the increase of venous osmolality At the maximal level of hyperosmolality encountered during chorda stimulation (mean $\approx$ 30 mOsm above control) the peak dilator response to hypertonic infusion averaged about 50 per cent and the steady state response about 20 per cent of the maximal papaverine induced dilatation in the gland It appears that an approximate quantitative evaluation of the role of tissue hyperosmolality in the functional hyperemia response can be reached by comparing at equal levels of hyperosmolality curves A and C (as regards the initial dilator response) and curves B and D (as regards the maintained dilator response) Such a comparison suggests that tissue hyperosmolality contributes significantly to both effects especially at low and moderate rates of chorda excitation

Atropine experiments

It was often stated in the past that atropine given in doses which abolish the secretion hardly at all interferes with the dilator response to chorda stimulation Recent studies however have shown that atropine can block or significantly reduce the dilator response at low to moderate frequency stimulation (Emmelin Garret and Ohlin 1968 Darke and Smaje 1972) especially with regard to the initial dilator phase These results were confirmed in a separate series of experiments (4 animals) in which the effects of atropine (200 μg/kg b w i v) on the functional hyperemia response and in addition on the hyperosmolality were studied These results are summarized in Fig 4 The resistance vessel response (initial and maintained) and the venous hyperosmolar response to graded chorda stimulation after atropine have here been expressed in per cent of the control stimulation effects before atropine (the latter = 100 per cent) It can be seen that the initial as well as the maintained hyperemia responses to chorda stimulation were completely blocked by atropine at the low stimulation rates and markedly reduced at 2 to 6 Hz At 10 and 20 Hz however atropine had little effect on the dilatation especially as regards the maintained dilator respon e The hyperosmolar response referring to the time period for the maintained hyperemia was much depressed by atropine at all rates of stimulation this was true also for the initial hyperosmolality response not shown in figure These results seem compatible with the hypothesis that tissue hyperosmolality i causally linked to the functional hyperemia response at low and moderate rates of chorda excitation

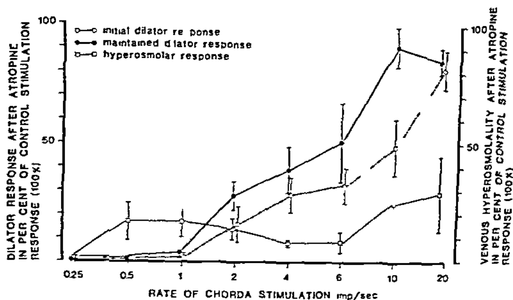


Fig 4 Diagram showing the effect of atropine (200 μg/kg b w i v) on the dilator response of the resistance vessels and on regional venous hyperosmolality in the submandibular gland during chorda stimulation. Each point in the curves refers to 4 separate observations

Discussion

The present study shows that parasympathetic activation of the submandibular gland causes a locally produced tissue hyperosmolality of considerable magnitude concomitant with the functional hyperemia response The osmolality was clearly raised during the initial dilator phase and reached a plateau level during the maintained steady state hyperemia response (Fig 1) Experimental tissue hyperosmolality in the resting gland produced by close arterial hypertonic infusion evoked a hyperemia response which resembled that observed during low and moderate rates of chorda stimulation (cf Fig 1 panel A and Fig 3) Comparison of the magnitude of the dilatation in the resistance vessels during chorda stimulation and during experimental hyperosmolality at similar increases of venous osmolality indicated that tissue hyperosmolality can contribute significantly to the functional hyperemia response in the gland this seemed to be the case both with regard to the initial and the maintained hyperemia phase and especially at low and moderate rates of nerve activation (Fig 2 This conclusion was supported by a separate series of experiments in which atropine was found to considerably depress both the hyperosmolality and the functional hyperemia response to chorda stimulation in the frequency range up to 6 Hz (Fig 4

The mechanisms by which the tissue hyperosmolality in the activated gland inhibits vascular tone may be similar to those previously described for the active skeletal muscle in which hyperosmolality was shown to be an important mediator of exercise hyperemia (Mellander *et al* 1967 Lundvall 1972) Those studies in

dicated that tissue hyperosmolality primarily develops in the intracellular compartment as metabolism rises during muscle contraction By release of diffusible substances from the activated striated muscle cells and by osmotic fluid (water) movement into these cells an increased osmolality ensues in the interstitial space *i e* in the immediate environment of the vascular smooth muscles The hyperosmolality as also evidenced by studies on isolated vascular smooth muscle (Mellander *et al* 1967 Johansson and Jonsson 1968 Arvill Johansson and Jonsson 1969 Jonsson 1970) leads to osmotic shrinkage of the vasculature This effect in turn causes relaxation and hence vasodilatation mainly due to inhibition of myogenic pacemaker activity It appears likely that the increased metabolism in the submandibular gland during activation via the tissue hyperosmolality can lead to the same train of events

In the mentioned studies on skeletal muscle the quantitative importance of tissue hyperosmolality for the functional vasodilatation was estimated by a similar comparative analysis to the one used in the present study (Fig 2) The conclusion was further based on several other types of experiments These included a critical analysis of possible errors inherent in the experimental approach related to rheological effects transcapillary fluid and osmolar exchange transintimal smooth muscle effects of hypertonic infusion etc It was concluded that such errors were relatively small and furthermore the effects tended to cancel out Repetition of a similar detailed analysis was therefore not considered essential for the present study and it is believed that the data in Fig 2 permit an approximate estimation of the role of tissue hyperosmolality for the functional vasodilatation in the submandibular gland

The present results thus indicate that tissue hyperosmolality contributes significantly to the functional hyperemia in the submandibular gland both in the initial and maintained phase of the vasodilatation The relative importance of this factor seems to be greatest at low and moderate rates of chorda excitation at excitation rates exceeding 6 imp/s its role is apparently fairly small It is interesting to note in this context that adequate stimulation of the salivary glands in intact dogs by food intake causes a chorda nerve discharge in the range of 2—8 Hz (Emmelin and Holmberg 1967) suggesting that higher stimulation rates may be supraphysiological It should be emphasized however that tissue hyperosmolality by no means is the sole causal factor for the functional hyperemia response not even at low rates of excitation it apparently acts synergistically with the previously described dilator control systems for the gland *i e* the cholinergic nervous and the bradykinin mechanisms

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References

ARVILL A B JOHANSSON and O JONSSON Effects of hyperosmolarity on the volume of vascular smooth muscle cells and the relation between cell volume and muscle activity *Acta physiol scand* 1969 75 484—495

BHOOLA K. D J MORLEY M SCHACHTER and L. H SMAJE Vasodilatation in the submaxillary gland of the cat. *J Physiol* (Lond) 1965 *179* 172—184
DARKE A. C and L. H SMAJE Dependence of functional vasodilatation in the cat submaxillary gland upon stimulation frequency *J Physiol* (Lond) 1972 *226* 191—203
EMMELIN N and J HOLMBERG Impulse frequency in secretory nerves of salivary glands *J Physiol.* (Lond) 1967 *191* 205—214
EMMELIN N J R. GARRETT and P OHLIN Neural control of salivary myoepithelial cells *J Physiol* (Lond) 1968 *196* 381—396
GAUTVIK, K. Studies on kinin formation in functional vasodilatation of the submandibular salivary gland in cats. *Acta physiol scand* 1970 *a* *79* 174—187
GAUTVIK K. The interaction of two different vasodilator mechanisms in the chorda tympani activated submandibular salivary gland *Acta physiol scand* 1970 *b* *79* 188—203
GAUTVIK K. Parasympathetic neuro-effector transmission and functional vasodilatation in the submandibular salivary gland of cats *Acta physiol scand* 1970 *c* *79* 204—215
GAUTVIK K. M S M HILTON and S H. TORRES Consumption of kininogen in the submandibular salivary gland when activated by chorda stimulation. *J Physiol* (Lond) 1970 *211* 49—61
HILTON S M and G P LEWIS The mechanism of the functional hyperaemia in the submandibular salivary gland. *J Physiol.* (Lond) 1955 *129* 253—271
HILTON S M and G P LEWIS The relationship between glandular activity bradykinin formation and functional vasodilatation in the submandibular salivary gland *J Physiol* (Lond) 1956 *134* 471—483
HILTON S M. and S H TORRES Selective hypersensitivity to bradykinin in salivary gland with ligated ducts *J Physiol* (Lond.) 1970 *211* 37—48
JOHANSSON B and O JONSSON Cell volume as a factor influencing electrical and mechanical activity of vascular smooth muscle *Acta physiol scand* 1968 *72* 456—468
JONSSON O Extracellular osmolality and vascular smooth muscle activity *Acta physiol scand* 1970 Suppl 359 1—48
KARPINSKI E S BARTON and M SCHACHTER, Vasodilator nerve fibres to the submaxillary gland of the cat. *Nature* (Lond) 1971 *232* 122—124
LUNDVALL. J Tissue hyperosmolality as a mediator of vasodilatation and transcapillary fluid flux in exercising skeletal muscle *Acta physiol scand* 1972 Suppl 379 1—142
MELLANDER S B JOHANSSON S GRAY O JONSSON J LUNDVALL and B LJUNG The effects of hyperosmolarity on intact and isolated vascular smooth muscle Possible role in exercise hyperemia. *Angiologica* 1967 *4* 310—322
MORLEY J M SCHACHTER and L. H SMAJE Vasodilatation in the submaxillary gland of the rabbit. *J Physiol* (Lond) 1966 *187* 595—602
MYER H A and C R. HONIG Influence of initial resistance on magnitude of response to vaso-motor stimuli *Amer J Physiol* 1969 *216* 1429—1436
SCHACHTER, M and S BEILENSON Mediator of vasodilatation in the submaxillary gland *Fed Proc* 1968 *27* 73—75
TERROUX K G P SEKELJ and A. S V BURGEN Oxygen consumption and blood flow in the submaxillary gland of the dog *Canad J Biochem* 1959 *37* 5—15

Acta physiol scand 1974 92 175—180
From the Departments of Pathology and Histology University of Uppsala Sweden

In Vitro Release of Corticosteroids from Adrenal Glands of Obese-Hyperglycemic Mice (Gene Symbol ob)

By

PETER NAESER

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Abstract

NAESER P *In Vitro release of corticosteroids from adrenal glands of obese hyperglycemic mice (gene symbol ob)* Acta physiol scand 1974 9? 175—180

The adrenal function of obese hyperglycemic mice of various ages was investigated by measurements of the corticosteroid release from hemisectioned adrenal glands incubated *in vitro* Glands from lean litter mates were used as controls The corticosteroid release was strongly stimulated by ACTH and this stimulated release remained constant throughout the experiments The ACTH effect was blocked when nitrogen was substituted for oxygen in the gas phase In all investigated age groups the adrenals of obese mice displayed a higher release of corticosteroids than those of the lean controls when incubated without addition of ACTH In the presence of ACTH the adrenal halves of obese mice showed a similar release of corticosteroids to those of the lean mice except for the 2 month-old group in which there was a smaller release from the adrenals of the obese animals Insulin had no stimulating effect on the corticosteroid release and was even slightly inhibitory in the absence of ACTH in the medium It is suggested that the increased size and steroid production of the adrenal glands of the obese-hyperglycemic mice may reflect a continuous functional stimulation such as could be exerted by ACTH

It has recently been shown that mice with the obese hyperglycemic syndrome (genotype *obob*) display a pronounced elevation of the corticosteroid hormone concentration in the blood (Naeser 1974) in addition to a number of previously described metabolic disturbances (*cf* Westman 1970) This observation conforms to previous data indicating enlarged adrenal glands in these animals (Marshall *et al* 1957 Hellerström *et al* 1962) and raises the question as to what extent the adrenal hyperfunction is related to the diabetic trait In approach to this problem it was found that adrenalectomy decreased the insulin resistance of obese hyperglycemic mice but on the whole had no effect on their overweight (Naeser 1973) It therefore appeared improbable that a hyperadrenocorticism *per se* was the

Key word obese hyperglycemic syndrome *obob* mice corticosteroid release adrenal glands *in vitro* ACTH-effect

primary cause of the syndrome Nevertheless, the adrenal enlargement and hormonal hypersecretion constitute integrated parts of the syndrome

In the present investigation the release of corticosteroids from the adrenal glands has been studied *in vitro* in order to see if an adrenal hypersection could be detected also in this experimental situation Furthermore this *in vitro* system was utilized in second secretion from the adrenal cells

Material and Methods

63 male obese hyperglycemic mice (genotype *obob*) and 66 lean litter mates of the same sex were used The animals belonged to a colony originating from The Jackson Laboratory Bar Harbor Maine U S A and bred at the Department of Histology University of Uppsala Sweden since 1959 as described previously (*cf* Naeser 1973)

All experiments were started between 8 and 10 a m The animals were killed by cervical fracture 14 h after deprivation of food The adrenal glands were removed immediately freed from adjacent fat and sectioned into 2 halves Each adrenal half was placed in a small glass vial (Keen *et al* 1963) and preincubated for 30 min in 500 µl Krebs Ringer bicarbonate buffer adjusted to pH 7 4 and containing 0 6 mg/ml glucose and 3 0 mg/ml bovine plasma albumin (Armour Pharmaceutical Company Ltd Eastbourne England) at 37 C with constant shaking (110 strokes/min) The gas phase was 95 % O_2 and 5 % CO_2 unless otherwise stated The preincubation medium was discarded and replaced by 500 µl fresh incubation medium of equal composition except for the addition of the 2 test substances i e insulin (Beef insulin Novo Industri A/S Bagsvaerd Denmark) and adrenocorticotropic hormone (ACTH) (Acton Ferring AB Malmö Sweden) in doses given below After an incubation period of 2 h the medium was collected in small plastic vials and either assayed immediately for its content of corticosteroids or frozen at −20 C for later determination of these hormones The wet weights of the incubated adrenal halves were measured on a torsion balance with a sensitivity of ±0 5 mg The error of the single weight determination was ±5 The corticosteroid content of the medium was assayed fluorometrically mainly according to DeMoor *et al* (1960) as described previously (Naeser 1974) The error of a single steroid determination varied from ±5 to 10 % on different days and the lower limit of detection was 1 µg corticosterone/100 ml The values were expressed as ng corticosterone released per mg wet tissue weight and 2 h

To test that the addition of ACTH to the incubation medium caused a maximal release of the steroids hemisectioned glands of 2 month old mice were incubated in medium to which was added ACTH at a concentration of either 180 mU/ml or 360 mU/ml In order to check that there was a constant release throughout the entire incubation period some experiments were performed in which the medium was changed after 2 h of incubation and the glands were further incubated for 2 h in the same kind of medium The amount of corticosteroids secreted into the medium during each of the two separate incubation periods were then compared Moreover the effect of ACTH was evaluated under anaerobic conditions by incubating hemisectioned adrenal glands of 1 month-old obese and lean animals in media with and without ACTH (180 mU/ml) with a gas phase of 95 % N_2 and 5 % CO_2

The effects of insulin (200 ng/ml) in the presence and absence of ACTH (180 mU/ml) were tested on hemisectioned adrenals from obese and lean mice 1 2 and 3 months old The incubations were performed either in a Krebs-Ringer bicarbonate buffer containing glucose and albumin as described above or in a similar medium to which was added either insulin (200 ng/ml) or ACTH (180 mU/ml) or both together

The results are expressed as mean values ± standard error of the mean (S E) Statistical significances were tested by Student's t test (Snedecor 1956) The error of the method was assessed from paired measurements by means of the formula $\sqrt{\frac{\sum D^2}{2n}}$ where D is the difference and n the number of pairs (Franko 1953)

Results

Table I shows that a maximal corticosteroid release was obtained from hemisectioned adrenals of 2 month-old obese mice already when incubated with the lower of the 2

TABLE I Corticosteroids (ng corticosterone/mg wet weight/2 h) released into the incubation medium from adrenal halves of 2 month-old obese mice incubated with different concentrations of ACTH (180 and 360 mU/ml) After the first 2 hour incubation period (A) the medium was changed and the glands were further incubated for 2 h in a similar medium (B) Numbers of observations within parentheses Mean values ± S E

Concentration of ACTH in the medium (mU/ml)	180	360
A	175±15 (8)	19[illegible]±29 (6)
B	160±15 (8)	175±21 (7)

tested ACTH concentrations Furthermore it can be seen that prolongation of the incubation for another 2 hour period after changing the incubation medium resulted in a similar corticosteroid release during each of the 2 incubations ($P > 0.05$)

The results obtained from incubations under anaerobic conditions are presented in Table II. Under these conditions addition of ACTH did not stimulate the release of corticosteroids from the adrenals either of obese or of lean animals ($P > 0.05$) However the adrenal glands of obese mice showed a higher basal release of corticosteroids than those of lean mice incubated without oxygen In fact the amount of corticosteroids liberated from the glands of the lean group was below the limit of detection ability of the present technique

Fig 1 shows the amounts of corticosteroids released by adrenals incubated in media containing insulin and ACTH in the various combinations In all groups investigated glands of obese mice incubated in a medium without addition of hormone released significantly higher amounts of corticosteroids than did adrenals of the lean controls ($P < 0.001$) Among the obese mice the 5 month-old group showed a higher basal release than the 2 month-old group ($P < 0.05$) The basal corticosteroid release of the 1 month old lean animals was higher than that of the 2 month-old group ($P < 0.05$) but did not differ from that of the 5 month old group ($P > 0.05$) Addition of insulin in the absence of ACTH was found to decrease the adrenal corticosteroid release in both 2 and 5 month-old obese mice ($P < 0.01$) No corresponding effect was seen in the 1 month-old obese mice or in the lean mice of any age ($P > 0.05$) Incubation in the presence of ACTH significantly increased the corticosteroid release from the adrenal glands of both obese

TABLE II Corticosteroids (ng corticosterone/mg wet weight/2 h) released into the incubation medium from adrenal halves of 1 month-old obese and lean mice incubated in an atmosphere of 95% N_2 and 5% CO_2 in the absence and presence of 180 mU/ml ACTH Numbers of observations within parentheses Mean values ± S E

Concentration of ACTH in the medium (mU/ml)	0	180
Obese mice	32±10 (6)	17±5 (6)
Lean mice	< 1 (6)	< 1 (6)

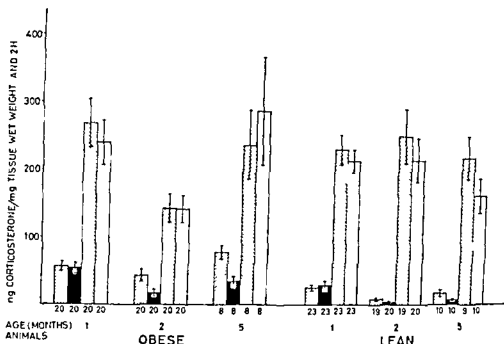


Fig 1 Corticosteroids (ng corticosterone/mg wet weight/2 h) released into the incubation medium from adrenal halves of 1 2 and 5 month old obese and lean mice incubated either in buffer alone [ ] or in buffer to which had been added insulin (200 ng/ml) ■ ACTH (180 mU/ml) [ ] or both together □ Numbers of observations are given below the bars Mean values ± S E

and lean mice in all groups investigated ($P < 0.01$) The amount of corticosteroids released seemed to be the same in obese and lean animals ($P > 0.05$) possibly except for the 2 month old obese mice which displayed a lower rate of corticosteroid secretion ($P < 0.05$) Addition of insulin in the presence of ACTH did not seem to influence the *in vitro release of corticosteroids* ($P > 0.05$)

Discussion

Several criteria were employed in order to assess the reliability of the present incubation technique Thus the rate of steroid secretion was strongly stimulated by ACTH and this stimulated release remained constant throughout a 4 hour incubation period These observations suggest that the regulation of the secretory process of the adrenal cortical cells was intact (Hechter *et al* 1951 Stone and Hechter 1954 Haynes 1957) and also indicates that a substantial part of the steroids recovered in the incubation medium did in fact consist of glucocorticoids (*cf* Carstensen *et al* 1961) Moreover the dependence of the release process on adequate cellular oxygenation (Simpson *et al* 1969) was confirmed by the observation that ACTH stimulation was blocked when nitrogen was substituted for oxygen in the gas phase The reason for the apparently increased steroid release

from the adrenals of the obese mice in the anaerobic situation remains obscure since oxygen is indispensable for the synthesis fo steroids (*cf* Simpson *et al* 1969) This observation possibly reflects leakage of preformed material from the cytoplasm of adrenal cells which were more heavily loaded with steroids than were those of the lean animals In fact we have observed a significantly higher corticosteroid content in the adrenals of obese mice than in those of the lean litter mates (Naeser unpublished data)

In all investigated age groups the adrenal halves of the obese mice exhibited a higher corticosteroid release than those of the lean litter mates when the incubations were performed without addition of ACTH Although this observation would seem to support the view that the hyperadrenocorticism of the obese animals is due to an uncontrolled hyperproduction of steroids by the adrenal cell it cannot be excluded that a raised circulating level of ACTH *in vivo* might affect these cells also *in vitro* Nevertheless addition of ACTH to the incubation medium produced the same pattern of response in the adrenal of both obese and lean animals. So far we have no explanation for the smaller *in vitro* effect of ACTH in the 2 month-old group

The possibility that the high circulating insulin concentration might affect the adrenal steroid production in the obese mice was tested by measuring the steroid release from adrenal halves which had been incubated with insulin in concentrations comparable with those observed *in vivo* (Westman 1968) The results suggest that insulin had no stimulating effect on the steroid release and was even slightly inhibitory in the absence of ACTH in the medium These data are also in agreement with the previous finding concerning the insulin sensitivity of the adrenocortical cells indicating that insulin had no effect on steroidogenesis from monolayer cultures of mouse adrenal cortical cells (Kowal and Fiedler 1968)

Previous observations on the steroid production *in vivo* in the obese mouse have shown that the adrenal response pattern to exogenous ACTH or stressful stimuli was generally qualitatively similar to that in the lean mouse (Naeser 1974) This is supported by the present finding that there were no major differences in the *in vitro* adrenal response to ACTH between obese and lean animals Thus on a per weight basis the adrenals of the two kinds of mice seemed to have the same maximal capacity for steroid release This would mean that the increased concentration of circulating steroid *in vivo* of the obese mice (Naeser 1974) is caused rather by an increase of the cortical volume (Hellerström *et al* 1962) than by a gross functional abnormality of the individual steroid producing cells Altogether the results of the present study *in vitro* and of previous investigations *in vivo* (Hellerstrom *et al* 1962 Naeser 1974) suggest that the increased size and steroid production of the adrenal glands of the obese hyperglycemic mouse are results of a continuous functional stimulation such as could be exerted by ACTH

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References

CARSTENSEN H B HELLMAN and S LARSSON Biosynthesis of steroids in the adrenals of normal and obese hyperglycemic mice *Acta Soc Med Upsalien* 1961 *66* 139—151

DE MOOR P O STEENO M RASKIN and A HENDRIKX Fluorometric determination of free plasma 11 hydroxycorticosteroids in man *Acta endocr* (Kbh) 1960 *33* 197—307

ERANKO O *Quantitative methods in histology and microscopic histochemistry* S Krager Publishers Basel — New York 1955 p 23

HAYNES R C JR and L BERTHET Studies on the mechanism of action of the adrenocorticotropic hormone *J biol Chem* 1957 *225* 115—124

HECHTER O A ZAFFARONI R P JACOBSEN H LEVY R W JEANLOZ V SCHENKER and G PINCUS The nature and the biogenesis of the adrenal secretory product *Recent Progr Hormone Res* 1951 *6* 215—241

HELLERSTROM C B HELLMAN and S LARSSON Some aspects of the structure of the adrenals in obese hyperglycemic mice *Acta path microbiol scand* 1962 *54* 365—372

KEEN H J B FIELD and I PASTAN A simple method for in vitro metabolic studies using small volumes of tissue and medium *Metabolism* 1963 *12* 143—147

KOWAL J and R FIEDLER Adrenal cells in tissue culture I Assay of steroid products Steroidogenic responses to peptide hormones *Arch Biochem* 1968 *128* 406—421

MARSHALL M B S B ANDRUS and J MAYER Organ weights in three forms of experimental obesity in the mouse *Amer J Physiol* 1957 *189* 343—346

NAESER P Effects of adrenalectomy on the obese hyperglycemic syndrome in mice (gene symbol *ob*) *Diabetologia* 1973 *9* 376—379

NAESER, P Function of the adrenal cortex in obese hyperglycemic mice (gene symbol *ob*) *Diabetologia* 1974 In press

SIMPSON E R D Y COOPER and R. W ESTABROOK Metabolic events associated with steroid hydroxylation by the adrenal cortex *Recent Progr Hormone Res* 1969 *25* 523—556

SNEDECOR G W *Statistical methods* fifth ed The Iowa College Press Ames Iowa 1956

STONE D and O HECHTER Studies on ACTH action in perfused bovine adrenals The site and action of ACTH in corticosteroidogenesis *Arch Biochem* 1954 *51* 457—469

WESTMAN S Development of the obese hyperglycemic syndrome in mice *Diabetologia* 1968 *4* 141—149

WESTMAN S The obese hyperglycemic syndrome in mice (*obob*) In *Les mutants pathologiques chez l animal* (Ed M Sabourdy) Editions du centre national de la recherche scientifique Paris 1970 155—176

Acta physiol scand 1974 92 181—194

From the Department of Physiology University of Goteborg Sweden

Comparison between Effects of Cyclic AMP, Dibutyryl Cyclic AMP and Luteinizing Hormone on Ovarian Glycolysis

By

H Herlitz L Hamberger and K Ahren

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Abstract

Herlitz H L Hamberger and K Ahren *Comparison between effects of cyclic AMP dibutyryl cyclic AMP and luteinizing hormone on ovarian glycolysis* Acta physiol scand 1974 92 181—194

Prepubertal rat ovaries were incubated in medium containing 5 5 mM glucose and various concentrations of cyclic AMP (cAMP) and dibutyryl cyclic AMP (dbcAMP) Glucose uptake and lactic acid production were stimulated in presence of 5 mM cAMP The effect was less pronounced with higher concentrations and 25 mM cAMP inhibited ovarian glycolysis compared to control ovaries A biphasic effect was also obtained with dbcAMP a dose dependent increase with lower concentrations (0 1—5 mM) and smaller effects with higher concentrations Luteinizing hormone (LH) stimulates ovarian glycolysis with a linear dose response relationship over an extremely wide range of concentrations (Hamberger and Ahren 1967) The stimulatory actions of dbcAMP and LH on ovarian glycolysis were augmented by low concentrations of aminophylline (0 5—1 mM) No additive effects were seen when LH and dbcAMP were used together in maximal stimulatory concentrations LH cAMP and dbcAMP were also compared as to their lipolytic actions on the ovary Lipolysis as judged by glycerol release was markedly enhanced by dbcAMP while the two other substances had only marginal effects When bovine serum albumin (acceptor for fatty acids) was present in the medium the dose response curve of the effect of dbcAMP on ovarian glycolysis was dramatically changed indicating an interaction between the lipolytic and the glycolytic effects of this compound

A stimulatory effect of LH on glycolysis in the isolated prepubertal rat ovary was shown in 1963 independently by Ahren and Kostyo (1963) and by Armstrong *et al* (1963) Various aspects of the cellular mechanisms of this effect as well as of the possible physiological significance of this LH action have since been analyzed in this laboratory (Ahren *et al* 1969 Ahren *et al* 1973) One of the aspects currently under investigation is whether this effect of LH is mediated partly or entirely *via* cyclic AMP (cAMP) as has been suggested for many other effects of this gonadotrophin (*e g* Marsh *et al* 1966 Tsafriri *et al* 1972 Channing and Seymour 1970) It has also been found that not only LH but also FSH can stimulate ovarian glycolysis (Hamberger and Ahren 1967) there is convincing proof that this effect of FSH is not due to a contamination of LH in the FSH preparation used (Hamberger and Ahren 1967 Ahren *et al* 1968 *a*)

One of the criteria which is usually used to prove that cAMP is involved as a mediator in the mechanism of action of a hormone is that addition of exogenous cAMP can mimic the effect of the hormone in the experimental system used (Sutherland *et al* 1968) One of the problems with such an analysis is that cAMP itself penetrates the cell membranes poorly This difficulty can usually be overcome by the use of various derivatives of cAMP *e g* dibutyryl cyclic AMP (dbcAMP) which penetrates the cell membrane more easily and which is also more resistant to enzymatic degradation by phosphodiesterase than is cAMP (Posternak *et al* 1962) It has however been reported in some experimental systems that cAMP and various derivatives of this molecule *e g* dbcAMP can produce effects which differ not only quantitatively but also qualitatively from that of cAMP (Sulimovici and Lunenfeld 1971 Pearlmutter *et al* 1973) This fact by necessity limits the usefulness of such substances in the experimental analysis of the second messenger theory

The present paper describes experiments in which the effect of LH on glycolysis in the isolated prepubertal rat ovary has been compared with the effect of cAMP and dbcAMP Preliminary experiments on glycerol release from ovaries incubated with LH cAMP and dbcAMP will also be reported

Material and methods

Animals Sprague Dawley rats purchased from Anticimex Ltd Stockholm Sweden were delivered to the animal quarters when 19—21 days old They were kept in rooms with constant temperature (24—26 C) and relative humidity (50—55 %) and exposed to controlled illumination of 14 h daily starting at 5 a.m The rats were given a semisynthetic diet (Gustafsson 1959) and water *ad libitum* Most rats were used for acute experiments when 24—26 days old with deprivation of food 20—24 h prior to the experiment A smaller group of rats were hypophysectomized when 26—28 days old and used for acute experiments 13—15 days later The latter group was not starved prior to the acute experiment

Removal and incubation of the ovaries The rats were killed by cervical fracture and the ovaries rapidly removed and placed in ice cold buffer Under the stereo-microscope they were rinsed of bursae and extraneous tissues and then incubated for various periods of time Krebs Ringer bicarbonate buffer pH 7 4 containing 5 5 mM glucose was used consistently The ovaries were collected in groups of 6 when both glucose uptake and lactic acid production were measured and in groups of 2 ovaries when only lactic acid production was measured The ovaries weighing 5—8 mg for the normal and 2—4 for the hypophysectomized rats were blotted on filter paper and placed in 10 ml Erlenmeyer flasks with 1 ml buffer Prior to the incubation the flasks were gassed with 95 % O_2—5 % CO_2 for 30 s sealed with tightly fitting rubber stoppers and placed in a gyratory water bath at 37 C Following incubation the flasks were placed on ice and the media withdrawn immediately The ovaries were removed from the flasks blotted on filter paper and rapidly weighed

Measurements and calculations Ovarian glucose uptake was determined by measuring the initial and final concentrations of glucose in the incubation media using the glucose-oxidase method (Saifer and Gerstenfeld 1958) Lactic acid production was determined enzymatically (Lundholm *et al* 1963) in the incubation medium at the end of the incubation period for reasons discussed elsewhere (Hamberger and Ahren 1967) Both glucose uptake and lactic acid production are expressed as μg/100 mg wet tissue weight. Glycerol was determined enzymatically in the incubation medium at the end of the incubation period according to Garland and Randle (1962) and expressed as μmoles/g wet tissue weight

Since it has been found that inhibition of protein synthesis can decrease the rate of glycolysis (Ahren *et al* 1968 *b*) the incorporation of leucine-^{3}H into ovarian protein was also studied in some experiments Ovaries which had been incubated in presence of 0 01 mM leucine ^{3}H for 2 h were homogenized in 1 ml of 10 % TCA The precipitate was spun down and washed once in 3 ml of 10 % TCA containing an excess of unlabelled amino acid and then heated for 15 min at 90 C in 3 ml fresh TCA The remaining precipitate was spun down and washed

TABLE I Effects of cAMP on glucose uptake and lactic acid production by isolated ovaries from normal and hypophysectomized[b] prepubertal rats

Addition to medium	Lactic acid production μg/100 mg × h			Glucose uptake μg/100 mg × h		
I Normal rats						
(controls)	68 3 ± 2 3	(17)		191 ± 5	(3)	
cAMP 5 mM	82 9 ± 5 7	(14)	$p < 0\ 05$	234 ± 17	(4)	$p < 0\ 05$
cAMP 25 mM	38 9 ± 4 4	(13)	$p < 0\ 01$	112 ± 6	(9)	$p < 0\ 05$
LH 1 μg/ml	110 0 ± 11 1	(4)	$p < 0\ 01$			
LH 25 μg/ml	135 4 ± 10 5	(4)	$p < 0\ 01$			
II Hypophysectomized rats						
(controls)	161 0 ± 9 1	(17)				
cAMP 1 mM	183 3 ± 12 2	(10)	NS			
cAMP 5 mM	238 3 ± 16 7	(12)	$p < 0\ 01$			
cAMP 25 mM	156 9 ± 19 3	(4)	NS			

[a] Ovaries from 24–26 day-old rats were incubated for 1 h in Krebs bicarbonate buffer pH 7 4 containing 5 5 mM glucose

[b] Rats were hypophysectomized when 26–28 days old and ovaries used for incubations 13–15 days later

All values are expressed as μg per 100 mg tissue and 1 h incubation time They are given as mean ± S E with the number of observation in parenthesis The significance of the difference from the control group is indicated by a p-value for each group

three times with ethanol ether chloroform (2 2 1) After the final wash the precipitate was dissolved in 1 ml of 1 N NaOH and boiled for 5 min The concentration of protein in the NaOH *solution was measured according to Lowry et al* (1951) The incorporation of radioactivity into the protein was determined by a modification of the Schoniger combustion technique (Schoniger 1955) as described in a previous paper (Ahren *et al* 1968 *b*) The radioactivity of the protein fraction is expressed as DPM/μg protein

Chemicals and hormones Adenosine 3 5-cyclic monophosphate (cAMP) puromycin di hydrochloride essentially fatty acid free bovine serum albumin and aminophylline were purchased from Sigma Chemical Co St Louis USA Two various preparations of N[6] 2 O dibutyryl adenosine 3 5 monophosphate (dbcAMP) were used one monopotassium salt from Calbiochem Ltd San Diego Calif USA and one monosodium salt from Boehringer Ltd Mannheim West Germany No quantitative or qualitative differences in effects of these two preparations were seen Leucine 4 5 ^3H with a specific activity (?00 μCi/μmol) was obtained from New England Nuclear Boston Mass USA Bovine LH preparations (NIH LH B3 B6 and B8) were supplied from the Endocrinology Study Section of the National Institutes of Health Bethesda, Md USA The activities of these hormones expressed in NIH LH S1 units/mg and the upper limit of the stated contamination with FSH in NIH FSH S1 units/mg (within brackets) are 1 0? (0 009) 0 89 (0 013) and 1 03 (0 05) respectively

Statistical procedures Mean values and standard error of the means (SEM) were calculated and the results compared by analysis of variance (with one criterion of classification) followed by Student Newman Keuls multiple range test (Woolf 1968) A p value of 0 05 or less was considered significant in this study

Results

Glucose uptake and lactic acid production

Influence of cAMP on ovaries from prepubertal and hypophysectomized rats In a first series of experiments 0 1 and 1 mM of cAMP was added to the incubation medium Glucose uptake and lactic acid production were determined after 1 h of incubation of the prepubertal rat ovaries No significant influence on either

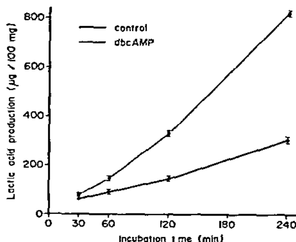


Fig 1 Lactic acid production by ovaries from prepubertal rats incubated for 30—240 min in the presence or absence of dbcAMP (1 mM) in the medium Two ovaries were incubated together in each flask in Krebs bicarbonate buffer containing 5 5 mM glucose The values are the mean ± S E The effect of dbcAMP is statistically significant at each point studied

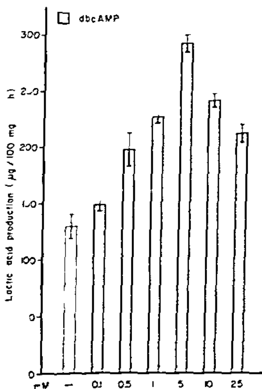


Fig 2 Effect of rious concentrations of dbcAMP on lacti acid production by ovaries from prepubertal rats The ovaries were incubated for 2 h at 3 in Kr bs bicarbonate buffer containing 5 5 mM glucose Each group represents the mean of 4 observations and standard error of the mean is indicated by a vertical l n on top of each bar All effects above 0 1 mM of dbcAMP are statist cally sign f cant in comparison to the contr l val

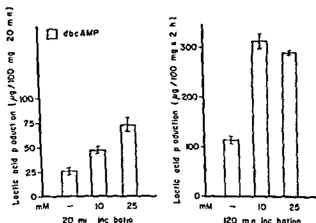


Fig 3 Effects of 10 and 25 mM dbcAMP on lactic acid production by isolated ovaries from prepubertal rats The ovaries were incubated for 20 and 120 min at 37 C in Krebs bicarbonate buffer containing 5 5 mM glucose After 20 min incubation the 25 mM value is higher than the corresponding 10 mM value while the relations are opposite after 120 min Each group represents the mean of 4 observations and standard error of the mean is indicated by a vertical line on top of each bar

parameter was however found until the nucleotide was added in a concentration of 5 mM With this concentration of nucleotide a significant stimulation was noted while a higher concentration (25 mM) caused a likewise significant but inhibitory effect (Table I) The effect of two concentrations of LH (1 and 25 μg/ml) tested under identical experimental conditions was quantitatively larger and no inhibition by higher concentrations was noted for this gonadotrophic hormone (Table I) (see also Hamberger and Ahren 1967) The lower part of Table I summarizes the influence of various concentrations of cAMP on lactic acid production by ovaries from hypophysectomized rats and principally the same pattern as for prepubertal rat ovaries was found

Influence of dbcAMP on ovaries from prepubertal rats Lactic acid production measured after various incubation periods in the presence or absence of 1 mM dbcAMP in the medium is shown in Fig 1 Lactic acid concentration in the medium increased almost linearly with time between 30 and 240 min Fig 2 shows lactic acid production by ovaries incubated for 2 h in presence of various concentrations of dbcAMP A significant stimulatory effect with a concentration of 0 5 mM a maximal stimulatory effect at 5 mM and a declining effect at higher concentrations were noted Comparison between the values of Fig 1 and 2 shows that the stimulation by 1 mM dbcAMP was more pronounced in the experiments presented in Fig 1 Similar variations in the degree of stimulation has earlier been found also with LH The pattern of the dose response curve was however always as demonstrated in Fig 2

Glucose uptake was measured in some experiments and this parameter was stimulated by 1 mM dbcAMP The increase was however, fairly modest (15 %) compared to the corresponding stimulation of lactate formation (75 %) At 5 mM this discrepancy was even more pronounced glucose uptake increased only [illegible] 11 % while lactate production was stimulated with 126 % With 10 mM dbcAMP in the medium lactate production was still increased with 86 % while glucose up-

TABLE II Effects of dbcAMP and LH on ovarian lactic acid production *in vitro* after preincubation and incubation with puromycin

Group	Lactic acid production μg/100 mg × 2 h Addition to m dium	
		puromycin
Control	175 5 ± 6 7	89 5 ± 8 9
dbcAMP 10 mM	319 8 ± 6 2	198 8 ± 7 2
LH 10 μg/ml	364 8 ± 2 1	164 7 ± 12 4

Ovaries from 24–26 day-old rats were first preincubated in medium containing puromycin di hydrochloride (500 μg/ml medium) for 15 min They were then transferred to new flasks and incubated for 2 h in Krebs bicarbonate buffer with or without puromycin (500 μg/ml) Both in absence and presence of puromycin the effects of dbcAMP and LH on lactic acid production are statistically significant ($p < 0 01$)

take was not stimulated at all (μg glucose taken up during 2 h per 100 mg ovary C = 385 0 ± 12 4 dbcAMP = 378 6 ± 12 4)

In the above mentioned experiments (Fig 2) in which the effect of 10 mM dbcAMP was less than that of 5 mM and the effect of 25 mM was even less the *ovaries were incubated for 2 h When a shorter incubation period (20 min)* was chosen (Fig 3) entirely different dose response curves were seen with the most marked stimulatory effect at a nucleotide concentration of 25 mM

Previous experiments (Ahren *et al* 1968 *b*) have shown that even in presence of the antibiotic puromycin which decreased the rate of glycolysis of the prepubertal rat ovray it was still possible to accelerate ovarian glycolysis with the addition of gonadotrophins This finding was confirmed in the present study (Table II), and it was also shown that dbcAMP stimulated ovarian lactic acid production in presence of puromycin (Table II)

Potentiation by aminophylline of the effects of LH and dbcAMP In order to further analyze whether the stimulatory effect of LH on glycolysis is mediated by cAMP experiments were performed with aminophylline in combination with the gonadotrophin and the nucleotide

TABLE III Effect of dbcAMP in combination with a high concentration of aminophylline on ovarian lactic acid production

Group	Addition to med um	Lactic acid production μg/100 mg × 2 h		
1	Control	164 8 ± 20 8	(4)	
2	Aminophylline 5 mM	88 0 ± 6 1	(4)	
3	Aminophylline 5 mM + dbcAMP 0 1 mM	132 7 ± 13 6	(4)	$p < 0 05$
4	Aminophylline 5 mM + dbcAMP 1 0 mM	147 9 ± 13 7	(4)	$p < 0 05$

Ovaries from 24–26 day-old rats were incubated for 2 h in Krebs bicarbonate buffer containing 5.5 *m*M glucose Number of observations in parentheses The p-values indicate the significance of the difference from the aminophylline group In presence of aminophylline the lactic acid levels are significantly lowered below the control levels ($p < 0 01$)

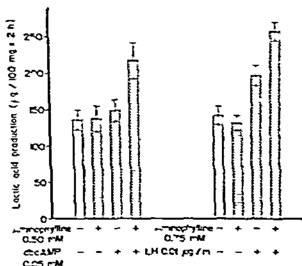


Fig. 4. Effects of low concentrations of LH (right) and dbcAMP (left) on ovarian lactic acid production in absence and presence of aminophylline. Ovaries from 24—26 day-old rats were incubated for 2 h in Krebs bicarbonate buffer containing 5.5 mM glucose. In presence of aminophylline the effect of both LH and dbcAMP are significantly augmented ($p < 0.05$). Each group represents the mean of 4 observations and S.E. is indicated by a vertical line on top of each bar.

In the first series of experiments the influence of aminophylline itself on lactate production of the ovary was examined. It was found that aminophylline in concentrations up to 1 mM had no significant influence on ovarian lactate production while in higher concentrations (2—10 mM) there was an inhibitory effect. The character of the inhibitory effect of high concentrations of the aminophylline was further analyzed by combining aminophylline 5 mM with dbcAMP. Also in presence of this high concentration of aminophylline dbcAMP had a clear stimulatory effect on ovarian lactate production (Table III).

Potentiation experiments were performed with low concentrations of aminophylline. Fig. 4 (left part) shows an experiment in which the addition of 0.50 mM aminophylline and 0.05 mM dbcAMP separately had no significant effect on lactate production. A combination of the two agents had a clearcut stimulatory effect. In a corresponding experiment with aminophylline and LH (Fig. 4, right part) the same type of potentiation was seen.

Table IV. Additivity between the effects of LH and dbcAMP on lactic acid production by prepubertal rat ovaries.

Exp. I Submax. conc.	Lactic acid prod. µg/100 mg × 2 h	Exp. II Maximal conc.	Lactic acid prod. µg/100 mg × 2 h
Control	118.7 ± 3.6	Control	135.3 ± 14.4
LH 0.05 µg/ml	179.4 ± 14.4	LH 10.0 µg/ml	334.2 ± 8.8
dbcAMP 0.5 mM	1[illegible]6.2 ± 13.4	dbcAMP 5 mM	283.4 ± 14.4
LH + dbcAMP	216.3 ± 5.2	LH + dbcAMP	343.4 ± 7.2

Ovaries from 24—26 day-old rats were incubated for 2 h in Krebs bicarbonate buffer, pH 7.4, containing 5.5 mM glucose. The number of observations in each group is five. Mean values ± S.E. are given. All groups significantly differ from the control groups ($p < 0.01$).

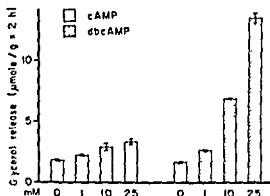


Fig. 5. Effects of two concentrations of LH and dbcAMP on lactic acid production by rat ovaries in absence and presence of 3 % albumin. The ovaries were incubated for 2 h in Krebs bicarbonate buffer containing 5.5 mM glucose. The mean values are connected in order to give a dose response line. There are 5 observations in each group.

Additive effects of LH and dbcAMP. The relationship between LH and dbcAMP was further characterized by adding the two substances together to the incubation medium (see Table IV). The maximal effective concentration of LH in terms of stimulation of lactic acid production has been determined to be 1000 µg/ml (Hamberger and Ahrén 1967). When a maximal concentration of dbcAMP was added together with this saturating concentration of LH no further increase of ovarian lactate production was seen. On the other hand the addition of dbcAMP to a low concentration of LH produced a clear additive stimulation above the effect of the hormone.

Amino acid incorporation into protein

Addition of LH to isolated prepubertal ovaries does not influence amino acid uptake or amino acid incorporation into ovarian protein (Noble and Kostyo 1963). Table V shows that leucine-³H incorporation was slightly stimulated when isolated ovaries were incubated in presence of 10 mM cAMP *in vitro*. At higher concentrations of the nucleotide a marked inhibition was seen with values 80—90 % below the control level. Using dbcAMP the stimulation appeared at a lower concentration and the inhibitory influence at high concentrations was very moderate.

TABLE V. Effects of cAMP and dbcAMP on leucine-³H incorporation into protein in isolated rat ovaries

cAMP mM	Leucine-³H incorporation DPM/µg protein	Significance of effect	dbcAMP mM	Leucine-³H incorporation DPM/µg protein	Significance of effect
0	3[illegible] ± [illegible] ([illegible])		0	342 ± 7 ([illegible])	
5	3[illegible]8 ± 17 (10)	NS		401 ± 23 (11)	p < 0.01
10	417 ± 12	p < 0.01	10	4[illegible] ± 17 (17)	p < 0.01
25	4[illegible] ± 12 ([illegible])	p < 0.01	25	2[illegible]3 ± 43 (11)	p < 0.01

Ovaries from 24—26 day-old rats were incubated for 2 h in Krebs bicarbonate buffer containing 5.5 mM glucose and 0.01 mM leucine-4,5-³H (2 µCi/ml). Number of observations are given in parentheses.

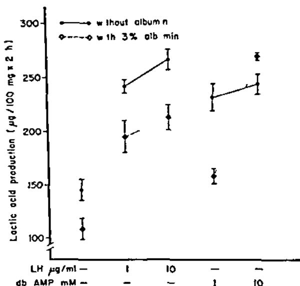


Fig 6 Influence of cAMP (left) and dbcAMP (right) on glycerol release from isolated prepubertal rat ovaries Ovaries from 24—26 day-old rats were incubated for 2 h in Krebs bicarbonate buffer containing 5.5 mM glucose and 3 % albumin Each group is the mean of 5 observations S E is indicated on top of each column The effect of cAMP is not significant at the lowest concentrations (1 mM) but in all other groups the mean values differ significantly from the control ($p < 0.01$)

Glycerol release

Influence by cAMP dbcAMP and LH on ovaries from prepubertal rats Fig 5 shows that both cAMP and dbcAMP increased the accumulation of glycerol in the incubation medium Dose response relationships were seen with the concentrations tested (1 to 25 mM) It can however also be seen from Fig 5 that dbcAMP had a much more pronounced effect than cAMP in this respect LH was also tested in these experiments and this hormone produced in some of the experiments an increase in glycerol accumulation of the same magnitude as that seen with cAMP However in other experiments no effect of LH was seen

In order to analyze whether the clear difference between LH and dbcAMP in their effects on glycerol release could explain the earlier mentioned difference in lactic acid production ovaries were incubated for 2 h in the absence and presence of 3 % albumin the latter serving as acceptor for fatty acids Two concentrations of LH and dbcAMP were tested in this system. Fig 6 shows that albumin in itself moderately reduced ovarian lactic acid production A pronounced change in the relation between the effects of the two concentrations of dbcAMP was produced (Fig 6) However albumin did not change the quantitative relation between the effects of the two LH concentrations

Discussion

Several observations of the present study are compatible with the theory that cAMP acts as an intracellular mediator for the glycolytic effect of LH on the prepubertal rat ovary 1) *addition of certain concentrations of cAMP or dbcAMP*

to the ovaries stimulated glucose uptake and lactic acid production, 2) the time course of the effect of 1 mM dbcAMP (Fig 1) was very similar to that produced by LH (Hamberger and Ahren 1967), 3) addition of low concentrations of aminophylline (a phosphodiesterase inhibitor) potentiated the effects of both LH and dbcAMP 4) no additive effects were seen when LH and dbcAMP were added in maximal stimulatory concentrations 5) both LH and dbcAMP stimulated ovarian lactate production in the presence of puromycin Recent observations that LH increases the activity of the adenylate cyclase (Lamprecht *et al* 1973) as well as producing a rapid and marked increase in the endogenous cAMP level in this type of rat ovaries are additional facts in accordance with this theory (Lamprecht *et al* 1973 Mason *et al* 1973 Ahren *et al* 1974) It cannot however, be excluded that the earlier observed decrease in ovarian ATP level after LH stimulation (Ahren *et al* 1968 *c*) is also an important factor in the regulation of ovarian glycolysis as has been discussed in a review article (Ahren *et al* 1969)

Even though addition of certain concentrations of cAMP and dbcAMP mimicked the effect of LH striking differences exist between the dose response curves of these three compounds After addition of LH a linear dose response curve is always seen over an extremely wide range of concentrations with more pronounced stimulation of glucose uptake and lactate formation when higher concentrations are added (Hamberger and Ahren 1967) High concentrations of cAMP on the other hand had a marked inhibitory effect on both glucose uptake and lactate formation while dbcAMP showed a reduction in its maximal stimulatory effect with high concentrations (Fig 2) Biphasic actions of both cAMP and dbcAMP have also been reported from other *in vitro* studies, *e g* from studies on glucose uptake in the isolated rat diaphragm (Chambaut *et al* 1969) In the present study the inhibitory concentration of cAMP (25 mM) on glycolysis also caused a marked inhibition of protein synthesis as measured by the rate of incorporation of leucine into ovarian protein (Table V) It has earlier been shown and it is confirmed in the present study that addition of puromycin to isolated ovaries in concentrations which blocks the protein synthesis decreased the rate of glycolysis markedly (Ahren *et al* 1968 *b*) It is therefore possible that the inhibitory effect of high concentrations of cAMP on glycolysis is secondary to an inhibition of protein synthesis High concentrations of dbcAMP (25 mM did not cause more than a slight decrease in rate of incorporation of amino acids into protein (Table V) and it seems unlikely that this very slight inhibition of protein synthesis would cause the marked decrease in rate of glycolysis as compared with lower concentrations of the nucleotide (5 – 10 mM) The high concentration of dbcAMP (25 mM) produced a pronounced release of glycerol and probably also of fatty acids into the incubation medium It is possible that the decreased rate of glycolysis is secondary to the lipolytic effect of 25 mM dbcAMP since it is well known that free fatty acids can inhibit the rate of glycolysis in many tissues *e g* the liver (see Lea and Weber 1968) Our experiments with albumin in the medium strengthen this explanation since albumin (*i e* an acceptor for free fatty acid) completely released the inhibitory effect of high concentrations

of dbcAMP Other mechanisms for this effect of albumin, can however not be completely excluded

Both quantitative and qualitative differences between the effects of cAMP and dbcAMP have been reported in the literature (Goodman 1969 Solomon *et al* 1970 Burke *et al* 1971) In the present experiments clear differences between the effects of these substances were also seen Firstly the stimulatory effect of dbcAMP on lactate production was superior to that of cAMP while the effects of these two substances were fairly equal on ovarian glucose uptake *i e* more lactic acid was produced in presence of dbcAMP than could be explained by the increased glucose uptake In this respect the effect of dbcAMP was also different from that of LH in all experiments with this gonadotrophin the ratio between glucose uptake and lactic acid production has been constant over a wide range of hormone concentrations (Hamberger and Ahren 1967) An increased utilization of endogenous substrates under the influence of dbcAMP could be one explanation to this discrepancy It has been shown that dbcAMP can activate glycogen phosphorylase and cause utilization of stored glycogen (Selstam and Ahren 1971) LH has however the same effect (Selstam and Ahren 1971), and the amounts of glycogen in the prepubertal rat ovary are comparatively small (Armstrong 1963) and can therefore only contribute marginally Other possible explanations for increased lactic acid production without a corresponding increase in glucose uptake are variations in the activity of the pyruvate dehydrogenase or in the cytoplasmic ratio of NADH/NAD Further experiments are required to analyse these possibilities *Secondly* cAMP produced in high concentrations a much more pronounced inhibition on lactate production glucose uptake and amino acid incorporation into protein than high concentrations of dbcAMP Thirdly ovarian lipolysis was far more stimulated by dbcAMP than by cAMP

One important question in studies with dbcAMP is whether this compound exerts its intracellular actions as such or whether deacylation to N^6 monobutyryl cAMP or cAMP is a requisite for biological effectiveness It was early reported for dog liver that dbcAMP has to be activated by removal of the butyryl group from the ribosyl residue before it becomes effective (Posternak *et al* 1962) and Kaukel *et al* (1972) obtained results indicating N^6 monobutyryl cAMP to be the true intracellular imitator of cAMP when dbcAMP was added to cultured HeLa cells In adipose tissue it was first reported that removal of butyryl groups from dbcAMP did not appear to be a prerequisite for biological activity (Blecher *et al* 1976) but significant deacylase activity has later on been found in adipose tissue (Blecher [illegible] Blecher and Hunt 1972) Recent studies have also shown that monobutyryl cyclic AMP (mbcAMP) but not dbcAMP can activate protein kinases from [illegible] heart (Miller *et al* 1973) as well as displace ^{3}H-cAMP bound [illegible] protein kinase preparation (Neelon and Birch 1973) It seems [illegible] that a deacylation of dbcAMP is a prerequisite for biological [illegible] ovary It was also necessary to test whether butyrate might [illegible] effects of dbcAMP No effects were however noted with [illegible]

Aminophylline in lower concentrations did not in itself significantly influence glucose uptake or lactic acid production but potentiated the stimulatory effect of both LH and dbcAMP. However, in higher concentrations aminophylline in itself had a clear inhibitory effect on lactic acid production. One possibility is that these high concentrations might inhibit ovarian protein synthesis as has been reported for isolated adrenal glands (Halkerston *et al.* 1966, Kitabchi *et al.* 1971). This would then lead to an inhibition of glycolysis similar to that found in presence of puromycin (Ahrén *et al.* 1968 *b*). The recent finding of Birmingham and Bartova (1973) is of interest in this connection. They reported that 10 mM theophylline inhibited the ACTH stimulated glycolysis in the isolated mouse adrenal gland, but it did not inhibit the stimulatory effect of 10 mM cAMP. They suggested that cAMP may have competed successfully for a receptor site shared with theophylline. In the present study it was found that dbcAMP still could stimulate glycolysis even in the presence of high concentrations of aminophylline.

In conclusion, the present experiments provide evidence in favour of the theory that cAMP is involved as a mediator for the glycolytic effect of LH. They illustrate in addition that exogenous addition of cAMP or dbcAMP can produce effects which do not correspond to the effects of a LH stimulated endogenous increase in cAMP in the ovary. A similar discrepancy between the effects of exogenous addition of cAMP and endogenous increase in cAMP level has been seen also in studies of amino acid transport in the prepubertal rat ovary. Addition of dbcAMP to isolated ovaries increased the rate of uptake of amino acids in the ovaries (Ahrén and Hamberger 1969) while addition of LH which produces a marked increase in ovarian cAMP level (Ahrén *et al.* 1974) did not influence ovarian amino acid transport. Caution must therefore be used in the interpretation of experiments using addition of cAMP or derivatives of this nucleotide to isolated ovaries, a conclusion which in principle can be drawn also from experiments on other *in vitro* systems, *e.g.* the thyroid (Burke *et al.* 1971) and the adrenals (Pearlmutter *et al.* 1973).

We wish to thank the Endocrinology Study Section of NIH for the generous supply of bovine LH (NIH-LH-B3, B6 and B8). The authors are also indebted to Dr Åke Hjalmarson who performed the hypophysectomies and to Dr Andre Beviz for valuable suggestions.

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References

Ahrén K. and J. I. Kostyo: Acute effects of pituitary gonadotrophins on the metabolism of isolated rat ovaries. *Endocrinology* 1963, *73*, 81—91.

Ahrén K., L. Hamberger and T. Perklev: Effects of human menopausal gonadotrophin and swine follicle stimulating hormone on lactic acid production by ovaries from prepubertal rats. *Endocrinology* 1968 *a*, *82*, 420—42?.

AHRÉN K L HAMBERGER and L RUBINSTEIN Influence of puromycin and anoxia on the effect of gonadotrophins on ovarian carbohydrate metabolism *Acta physiol scand* 1968 *b* 74 79—90

AHRÉN K A BEVIZ L HAMBERGER and L LUNDHOLM Mechanism for the action of luteinizing hormone (LH) on the carbohydrate metabolism of the prepubertal rat ovary *Acta physiol scand* 1968 *c* 73 23A—24A

AHRÉN K L HAMBERGER and L RUBINSTEIN Acute *in vivo* and *in vitro* effects of gonadotrophins on the metabolism of the rat ovary In *The Gonads* Ed McKerns K W Appleton Century Crofts New York 1969 327—354

AHRÉN K and L HAMBERGER Effect of cyclic 3 5 AMP on ovarian amino acid transport. *Acta physiol scand* 1969 *Suppl 330* 75

AHRÉN K L HAMBERGER, H HERLITZ T HILLENSJO L NILSSON T PERKLEV and G SELSTAM Aspects of the mechanism of action of gonadotrophins In *The endocrine function of the human testis'* Eds James V H T M Serio and L Martini. Academic Press New York 1973 Vol I 251—272

AHRÉN K H HERLITZ L NILSSON T PERKLEV S ROSBERG and G SELSTAM Gonadotrophins and cyclic AMP in various compartments of the rat ovary In *Hormones and Polypeptides Vol 7* Eds Li C H and Moudgal N R Academic Press New York 1974 In press

ARMSTRONG D T Stimulation of glycloytic activity of rat corpus luteum tissue by luteinizing hormone *Endocrinology* 1963 *72* 908—913

ARMSTRONG D T R KILPATRICK and R. O GREEP *In vitro* and *in vivo* stimulation of glycolysis in prepubertal rat ovary by luteinizing hormone *Endocrinology* 1963 *73* 165—169

BIRMINGHAM M K and A BARTOVA, Effects of calcium and theophylline on ACTH and dibutyryl cyclic AMP-stimulated steroidogenesis and glycolysis by intact mouse adrenal glands *in vitro Endocrinology* 1973 *92* 743—749

BLECHER M J T ROANE and P D FLYNN Metabolism of dibutyryl cyclic AMP in adipose tissue *Fed Proc* 1970 *29* 661

BLECHER M Biological effects and catabolic metabolism of 3 5 -cyclic nucleotides and derivatives in rat adipose tissue and liver *Metabolism* 1971 *20* 63—77

BLECHER M and N H HUNT Enzymic deacylation of mono- and d butyryl derivatives of cyclic adenosine 3 5 monophosphate by extracts of rat tissues *J biol Chem* 1972 *247* 7479—7484

BURKE G K KOWALSKI and S YU Effects of 3 5 -cyclic nucleotides on membrane transport in isolated thyroid cells *Acta endocr* (Kbh) 1971 *68* 183—195

CHAMBAUT A M D EBOUÉ BONIS J HANOUNE and H. CLAUSER, Antagonistic actions between d butyryl adenosine 3 5 -cyclic monophosphate and insulin on the metabolism of the surviving rat diaphragm. *Biochem biophys Res Commun* 1969 *34* 283—289

CHANNING C P and J F SEYMOUR Effects of dibutyryl cyclic 3 5 AMP and other agents upon luteinization of porcine granulosa cells in culture *Endocrinology* 1970 *87* 165—169

GARLAND P B and P J RANDLE A rapid enzymatic assay for glycerol *Nature* (Lond) 1962 *196* 987—988

GOODMAN H M The effects of insulin on lipolysis evoked by cyclic AMP and its dibutyryl analogue *Proc Soc exp Biol (N Y)* 1969 *130* 97—100

GUSTAFSSON B E Lightweight stainless steel systems for rearing germfree rats *Ann N Y Acad Sci* 1959 *78* 17—28

HALKERSTON I D K. M FEINSTEIN and O HECHTER, An anomalous effect of theophylline on ACTH and adenosine 3 5 monophosphate stimulation. *Proc Soc exp Biol (N Y)* 1966 *122* 896—900

HAMBERGER L and K AHRÉN Effects of gonadotrophins *in vitro* on glucose uptake and lactic acid production of ovaries from prepubertal and hypophysectomized rats *Endocrinology* 1967 *81* 93—100

KAUKEL E K. MUNDHENK and H HILZ N^6 monobutyryladenosine 3 5 monophosphate as the biologically active derivative of dibutyryladenosine 3 5 monophosphate in HeLa S3 cells *Eur J Biochem* 1972 *27* 197—200

KITABCHI A E D B WILSON and R. K. SHARMA Steroidogenesis in isolated adrenal cells of rat. *Biochem biophys Res Commun* 1971 *44* 898—904

LAMPRECHT S A. U ZOR A TSAFRIRI and H R. LINDNER *J Endocr* 1973 *57* 217—233

LEA M A and G WEBER Role of enzymes in homeostasis Inhibition of the activity of glycolytic enzymes by free fatty acids. *J biol Chem* 1968 *243* 1096—1102

LOWRY O H N J ROSEBROUGH A L FARR and R. J RANDALL Protein measurement with the Folin phenol reagent. *J biol Chem* 1951 *193* 265—275

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From the Institute of Medical Pharmacology University of Uppsala, Sweden

Bile Acids as Inhibitors of the Liver-like Anion Transport System in the Rabbit Kidney, Uvea and Choroid Plexus

By

ERNST H BARANY

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Abstract

BARANY E H *Bile acids as inhibitors of the liver like anion transport system in the rabbit kidney uvea and choroid plexus* Acta physiol scand 1974 *92* 195—203

Slices of rabbit kidney cortex pieces of lateral choroid plexus and of ciliary body with iris were incubated in a potassium rich medium containing iodipamide and o-iodohippurate labelled with different isotopes as test substances for the L and the H system respectively Cholic deoxycholic chenodeoxycholic lithocholic and dehydrocholic acids and their glycine and taurine conjugates were added as inhibitors and their ability to suppress iodipamide uptake preferentially compared The most selective inhibitor was free chenodeoxycholic acid followed by deoxycholic Considerable inhibition was caused at 100 μM by the dihydroxy acids At this concentration uptake of quaternary cations as toxicity indicators was hardly affected Non ionic detergents were unselective inhibitors Thus bile acids have affinity for the liverlike anion transport system in the kidney eye and choroid plexus

There are 2 overlapping transport systems for organic anions in kidney cortex ciliary processes and choroid plexus of a variety of species the hippurate or H system and the liver like or L system (Barany 1972) The two systems are overlapping in the sense that until now no substance has been found that saturates the one without affecting the other or that is taken up by only the one But there are H selective and L selective substances, even if selectivity is not absolute The systems have been studied by using two such test substances labelled with different iodine isotopes o iodohippurate (Hippuran®) that has higher affinity to the H system and the cholangiographic agent iodipamide (Biligrafin®) that has higher affinity to the L-system

Besides to iodipamide the L system has affinity to a variety of liver excreted sub stances hence the name (Barany 1973 b) In a search for physiological compounds possibly transported by the system rabbit gall bladder bile was tested in vitro as an inhibitor of the uptake of the two test substances into rabbit kidney cortex slices It was found to be rather L-selective (Barany 1973 a) which indicates the presence

bile of something that fits the L system Since this could be the bile salts a variety of bile acids have now been tested as inhibitors of the two transport systems in rabbit tissues A few experiments were also done in rat and cat kidney All bile acids conjugated or not, turned out to be more or less I selective, some were very selective indeed Since they are also toxic, however, a special study was made of how toxicity affects their selectivity

Methods

The methods used were very similar to those described (Bárány 1972) but the present protocol differs from the previous one by putting more emphasis on exactly identical incubation times and on making sure that slices of both kidneys contribute equally to the results at every concentration of every inhibitor

Rabbits of both sexes almost all albinos were used They were on conventional rabbit pellets and not starved before the experiment. The animals were anesthetized with urethane both kidneys removed decapsulated cut transversely into halves and put into ice cold saline Without delay they were then sliced to 0 3—0 5 mm in a modified Stadie Riggs slicer

The slices were immediately put into 18 open Petri dishes with very shallow basal medium at 37 exposed to the air Slices from half of the first kidney were put singly into dish 1—9 in that order slices from half the other kidney then into dishes 9—1 The other two half kidneys were distributed similarly into dishes 10—18 The two kidneys were sliced by one person while a second person separated outer cortex from juxtamedullary cortex and medulla in the Petri dishes Outer cortex slices from each dish were then put into one of 18 incubation bottles according to a time schedule that allowed exactly 20 min incubation while the sojourn in the Petri dishes was variable about 4—16 min The eyes were enucleated in rapid succession iris with ciliary body put into Petri dishes and cut radially into 6 pieces per eye This represents anterior uvea or eye

Finally the animal was killed by intracardiac urethane and the two lateral choroid plexi excised each cut into two pieces and also put into Petri dishes Plexus usually stayed there for only 2—4 min eyes 5—15 min before they were put into incubation bottles The bottles contained 5 ml solution and 70 ml room air They were shaken at 37 C Control bottles were shaken at 0 C

Tissues were removed from the bottles after exactly 20 min blotted between folds of hardened filter paper and very quickly weighed on Al foil on torsion balances The wet weight of the pieces was around 20 mg for cortex 12 mg for uvea and 7 mg for plexus They were then dried and counted in a γ spectrometer Two 50 μl samples of incubation medium taken after the incubation were put onto small pieces of cellulose sponge weighing about 15 mg and then treated as two tissue samples In experiments with ^{14}C labelled test compounds tissue digestion and conventional liquid scintillation techniques were used

As a rule 18 incubation bottles were used each containing 2 cortex slices The 12 pieces of uvea and 4 of plexus were distributed according to the needs for information

The basal incubation medium has been described (Bárány 1972) it is K rich (37 mM) and phosphate buffered to pH 7 4 In order to prevent uptake of free radioactive iodide it always contained 1 7 mM cold NaJ The test compounds I^{131} o-iodohippurate and I^{125} iodipamide were used in micromolar concentrations usually even less The bile salts to be tested as inhibitors were usually freshly dissolved in incubation medium *but some were made* up as stock solutions in absolute ethanol In these experiments the same amount of ethanol 0 05 ml/5 ml medium was added to all bottles Each run using one rabbit contained at least 2 often 4 uninhibited bottles and one ice incubated The tissue medium *ratios were corrected* for the ice cold uptake and then expressed as relative uptake as percent of the average T/M in the uninhibited bottles Thus the same control bottles were used for several concentrations of any inhibitor and in fact for several inhibitors Since the control bottles contributed to variability the distribution of the relative uptake figures is skewed while the distribution of their logarithms should be more symmetrical Averages *between results from* different experiments and their standard errors were therefore formed after logarithmation

Materials ^{125}I iodipamide ^{75}Se selenite and ^{75}Se selenomethionine were from the Radiochemical Centre Amersham ^{131}I-o-iodohippurate from AB Atomenergi Studsvik Cold iodipamide from Schering AG Berlin as Biligrafin Forte The bile acids were from several sources as listed below *They were used without further purification* ^{14}C *Cetiprin (emeprontum* Br) was a gift from Mr S Carlsson AB Recip Stockholm ^{14}C tetraethylammonium *bromide*

Acids	Form	Source	Grade	Lot
Cholic	Na	S	99 %	15B-0780
Glyco —	Na	C	A	310063
Tauro —	Na	C	A	800010
Deoxycholic	H	C	A	200829
Glyco —	Na	C	A	310088
Tauro —	Na	C	A	200742
Chenodeoxycholic	H	C	A	110075
Glyco —	Na	C	A	210181
Tauro —	Na	C	A	310013
Lithocholic	H	C	A	200657
Glyco —	H	D		
Tauro —	H	D		
Dehydrocholic	H	P	U S P	R69399
Glyco —	Na	C	A	210707
Tauro —	Na	C	A	100161

C = Calbiochem
D = Dr H Danielsson Karolinska Institutet
P = Pharmacia
S = Sigma

(TEA) was from New England Nuclear Boston and ¹²⁵I-o-iodobenzyltrimethylammonium a gift from Dr Ray Counsell University of Michigan Triton® X 100 was practical grade the Tweens ® were medicinal grade

Results

As a start the way of plotting the data will be explained Its rationale has been presented (Barany 1973 a) The difficulty to be overcome is that we have to make do without fully selective test compounds

Fig 1 A shows how the simultaneous uptake of iodipamide and iodohippurate (hippuran) in rabbit kidney slices is differentially inhibited by increasing concentrations of pooled rabbit bladder bile in the medium. (Same data as in Fig 5 Barany 1973 a) Iodipamide uptake is depressed before uptake of iodohippurate Fig 1 B shows the same data (except the point at 5 % bile) plotted on a logarithmic grid with percent iodipamide uptake on the abscissa and percent iodohippurate uptake on the ordinate The straight line D is a diagonal if an inhibitor were completely unspecific it would depress both uptakes equally and the resulting points would fall along D This would be so in spite of the fact that neither hippuran nor iodipamide is taken up exclusively by the L- or the H system Line I is the line obtained with cold iodipamide in increasing concentrations (data from Barany 1972) This is a reference line to indicate if an inhibitor is more or less L-selective than iodipamide More selective inhibitors yield points to the left of this line

The points connected by the line B are the same data as plotted in 1 A They show that rabbit bile is almost as L-selective as iodipamide at the three uppermost points while it may be less selective at 5 % bile which leaves only 8 percent of

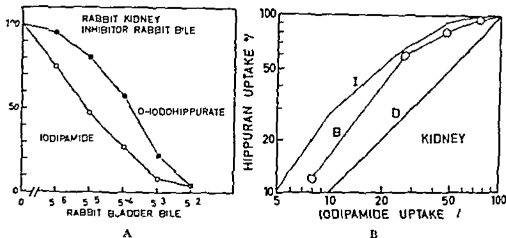


Fig 1 Inhibition by bile of o-iodohippurate (hippuran) and iodipamide uptake plotted in two ways A. Abscissa Bile concentration ordinate Uptake as percent of uninhibited B Same data plotted so as to show the selectivity of bile as an inhibitor Uptake as percent of uninhibited on both axes The curved line labelled I represents iodipamide as an inhibitor and appears also in Fig 2 and 3 Further explanation see text.

iodipamide uptake and 12 of iodohippurate These low figures are rather uncertain and the loss of specificity at high concentration is not proved beyond doubt by these data

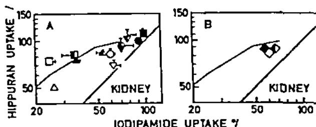
Rabbit bladder bile is about 200 mM with respect to bile salts, of which a little more than 100 mM is glycodeoxy and about 35 mM glycohenodeoxycholate (data of Johnson and Kalant 1972) Thus the concentration of total bile acids in Fig 1 A ranges from 13 μM to 8 mM and in 1 B from 13 μM to 1 6 mM

Let us now turn to pure bile acids plotted in the way of Fig 1 B

Fig 2 A and B show results with the listed bile acids at 100 μM (A) and 67 μM (B)

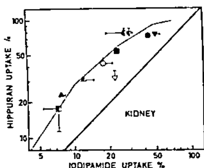
It is immediately apparent that all bile acids tested have yielded points on the L-selective side of the diagonal Lithocholic acid and its two conjugates are close to their saturated concentration in Fig 2 B Those of the others that are soluble enough have been tested also at 1 mM (Fig 3) At this concentration the points are better separated and most of them fall close to the iodipamide reference line Free dehydrocholic acid is an exception it is markedly less L-selective than the others both in Fig 2 A and 3 The most L-selective of all is free chenodeoxycholic acid which at 100 μM depresses iodipamide uptake to 24 % while hippuran uptake still is 74 % Since a considerable part of iodipamide uptake maybe 20 % or more is by the H system these figures would indicate almost total inhibition of the L-system

Fig 4 shows how uptake in choroid plexus is affected by 100 μM bile acids (the litho group at 67 μM Chenodeoxycholic and its glycine conjugate are the most L-selective, followed by the deoxy group The same holds for Fig 5 data for



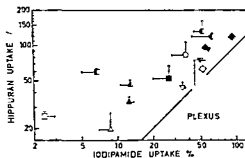
	CHOLIC	DESOXY	CHENO	DEHYDRO	LITHO
UNCONJ	○ 5	△ 6	□ 6	▽ 7	◇ 4
GLYCO	◐ 5	▲ 8	◧ 4	▼ 4	◆ 7
TAURO	● 5	▲ 6	■ 4	▼ 4	◆ 7

Fig 2 A Selectivity of bile acids at 100 μM as inhibitors of hippuran and iodipamide uptake in slices of rabbit kidney cortex B Same for lithocholic acid and conjugates at 6 7 μM The error indicators (bars) represent 1 S E some times they are hidden in the symbols The number of animals is given besides the explanations of the symbols each animal contributed at least 2 kidney cortex slices with bile acid and 4 controls per inhibitor



	CHOLIC	DESOXY	CHENO	DEHYDRO
UNCONJ	○ 4			▽ 7
GLYCO	◐ 4	▲ 4	◧ 4	▼ 4
TAURO	● 6	▲ 8	■ 4	▼ 4

Fig 3



	CHOLIC	DESOXY	CHENO	DEHYDRO	LITHO
UNCONJ	○ 4	△ 2	□ 2	▽ 5	◇ 2
GLYCO	◐ 4	▲ 4	◧ 2	▼ 2	◆ 2
TAURO	● 3	▲ 3	■ 2	▼ 1	◆ 1

Fig 4

Fig 3 Selectivity of bile acids at 1 mM as inhibitors of hippuran and iodipamide uptake in slices of rabbit kidney cortex In other respects the legend of Fig 2 holds

Fig 4 Selectivity of bile acids at 100 μM as inhibitors of hippuran and iodipamide uptake in lateral choroid plexus of rabbits One control and 1 inhibited piece per inhibitor and animal If n > 1 a lacking standard error bar indicates that standard error is hidden by the symbol. In other respects the legend of Fig 2 holds

anterior uvea The eye preparation used contains a much lower proportion of active tissue than plexus and kidney cortex this causes rather more scatter and larger standard errors Nonetheless it is evident that in all three tissues the L-system is more depressed than the H system by bile acids as a group and that different bile acids have different selectivity

Glycodeoxycholic acid the main bile acid in rabbit bile was somewhat less L-selective than glycochenodeoxycholic

A few experiments were made with rat and cat kidney only one experiment per inhibitor (2 rats 1 cat) The picture evidently is quite similar to that in the rabbit

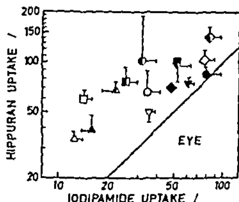


Fig 5 Selectivity of bile acids at 100 μM as inhibitors of hippuran and iodipamide uptake in anterior uvea (iris + ciliary processes) of rabbits Two control and at least 1 inhibited piece per inhibitor and animal In other respects the legend of Fig 2 holds

	CHOLIC	DESOXY	CHENO	DEHYDRO	LITHO
UNCONJ	○ 5	△ 6	□ 5	▽ 7	◇ 4
GLYCO	◐ 3	◭ 5	◧ 4	▼ 4	◆ 4
TAURO	● 3	▲ 5	■ 2	▼ 2	◆ 2

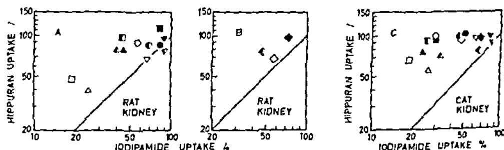


Fig 6 A Selectivity of bile acids at 100 μM as inhibitors of hippuran and iodipamide uptake in slices of rat kidney cortex B The same for lithocholic acid and conjugates at 6 7 μM C The same concentrations but cat renal cortex Only one experiment (2 exptl 4 control slices per point) Symbols as in Fig 2

(Fig 6 A B C) Here too chenodeoxycholic and its conjugates are strikingly L selective closely followed by the deoxy group

Now bile acids are cytotoxic substances (see Heaton 1972 for references) and the dihydroxy acids deoxy and chenodeoxycholic are much more toxic than cholic or dehydrocholic Could the apparent high selectivity of the dihydroxy acids be in fact an expression of their toxicity?

One aspect of their toxicity is detergency Could the cells or the transport system responsible for the L system be more vulnerable to detergent action than those belonging to the H system? Fig 7 shows an experiment with 3 non ionic detergents In sufficient concentration they all depress uptake but the depression is rather unselective the points fall close to the diagonal even if Triton® X 100 tends to be slightly L-selective and Tween® 20 slightly H selective The same holds for anterior

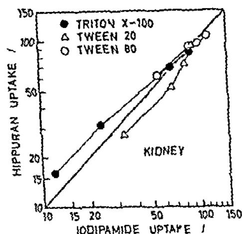


Fig 7 Lack of selectivity of non ionic deter gents as inhibitors of hippuran and iodipam ide uptake in slices of rabbit kidney cortex Inhibitors all at 10^{-6}—10^{-3} v/v Only 1 expt (2 exptl 4 control slices per point)

urea (not shown) Thus it is unlikely that simple detergency is responsible for or greatly affects the L-selectivity of all the bile acids

Bile acids could be toxic in other ways If this toxicity were due to the extracellular presence of the acids it probably would be unselective, but it cannot be excluded that different cell groups in the kidney cortex differ in their sensitivity Then since the proportion between H and L-systems is not equal in all cells in the rabbit kidney cortex (Farany 1975 a) the bile acids could conceivably exert a selective toxic action which in itself has nothing to do with their affinity to the L system as a transport system It is very improbable however, that in the ciliary processes and the choroid plexus there would exist a differential sensitivity to the toxic action similar to that of the cells of the kidney cortex Since L-electivity of the bile acids was evident also in the ciliary processes and the choroid plexus it seems improbable that the L selectivity observed in the kidney could be due to a selective toxic action Nonetheless it seemed worth while to investigate at which concentration bile acid toxicity becomes evident under our condition

In the complex situation outlined it is improbable that any single test compound could be a really relevant toxicity indicator Accordingly five toxicity indicator compounds selected so as hopefully not to be transported by the H and L systems were tried in kidney cortex experiments identical with the standard iodipamide iodohippurate ones The results were disappointing

The compounds tested were ^{14}C tetraethylammonium ^{14}C Cetiprin and ^{125}I trimetyl p iodobenzylammonium all quaternaries ^{75}Se elenomethionine and ^{75}Se selenite The uptake of selenomethionine was depressed by high concentrations of hippurate as well a iodipamide this aminoacid and presumably others would thus not be suitable as test compounds for unspecific toxicity of substances that affect the organic anion systems Selenite uptake too was depressed by high concentrations of iodipamide The quaternaries however were quite insensitive to iodipamide concentrations which completely saturate both the H and the L-system When

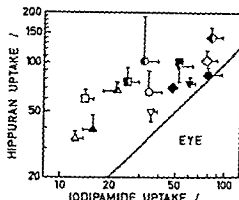
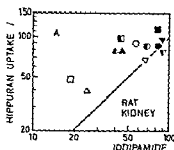
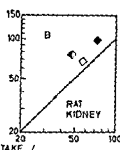


Fig 5 Selectivity of bile acids at 100 μM as inhibitors of hippuran and iodipamide uptake in anterior uvea (iris + ciliary processes) of rabbits Two control and at least 1 inhibited piece per inhibitor and animal In other respects the legend of Fig 2 holds

	CHOLIC	DESOXY	CHENO	DEHYDRO	LITHO
UNCONJ	○ 5	△ 6	□ 5	▽ 7	◇ 4
GLYCO	◐ 3	▲ 5	◧ 4	▼ 4	◆ 4
TAURO	● 3	▲ 5	■ 2	▼ 2	◆ 2





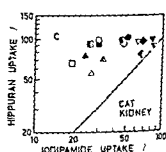


Fig 6 A Selectivity of bile acids at 100 μM as inhibitors of hippuran and iodipamide uptake in slices of rat kidney cortex B The same for lithocholic acid and conjugates at 6 7 μM C The same concentrations but cat renal cortex Only one experiment (2 exptl 4 control slices per point) Symbols as in Fig 2

(Fig 6 A B C) Here too chenodeoxycholic and its conjugates are strikingly L selective closely followed by the deoxy group

Now bile acids are cytotoxic substances (see Heaton 1972 for references) and the dihydroxy acids deoxy and chenodeoxycholic are much more toxic than cholic or dehydrocholic Could the apparent high selectivity of the dihydroxy acids be in fact an expression of their toxicity?

One aspect of their toxicity is detergency Could the cells or the transport system responsible for the L system be more vulnerable to detergent action than those belonging to the H system? Fig 7 shows an experiment with 3 non ionic detergents In sufficient concentration they all depress uptake but the depression is rather unselective the points fall close to the diagonal even if Triton® X 100 tends to be slightly L selective and Tween® 20 slightly H selective The same holds for anterior

cells of the ileum Thus the special L-selectivity of chenodeoxycholic acid is suspiciously large The selectivity implies that the acid depresses the L-system much more than the H system If in fact it only depresses the selectivity is real whatever its explanation—competition, toxicity or something else But if the acid at the concentration used, were to stimulate iodohippurate uptake by one group of cells while it depresses uptake by most of them the global depression of iodohippurate would be smaller and a super selectivity simulated Clearcut stimulation of iodohippurate uptake is rarely observed with bile acids but frequently with their close relatives, fusidic acid and derivatives (Barany unpublished) Stimulation was seen mainly in cortex slices very rarely in plexus or anterior uvea. Thus the L-selectivity of some bile acid in kidney experiments may be exaggerated but there can be no doubt that they are in fact rather L-selective

Bile acids are large organic anions and this fact alone should make them L-selective (Barany 1973 b) That they do have affinity to the H and L-systems does not in itself imply that they are efficiently transported by these systems One most efficient bile acid transport system that of the ileum is definitely different from the H and L-systems it has no affinity to iodohippurate or iodipamide (Barany unpublished) But in fact cholate and glycocholate are as mentioned accumulated by plexus eye and kidney cortex of several species and part of this accumulation can be inhibited by hippurate or iodipamide Furthermore cholate is transported out of the CSF in the rat and this transport is inhibited by iodipamide (Lundberg 1974) These results will be dealt with in forthcoming publications.

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Referens

Barany E H., Inhibition by hippurate and probenecid of in vitro uptake of iodipamide and o-iodohippurate. A composite uptake system for iodipamide in choroid plexus kidney cortex and anterior uvea of several species *Acta physiol scand* 1972 *86* 12—27

Barany E H. The liver like anion transport system in rabbit kidney uvea and choroid plexus I Selectivity of some inhibitors direction of transport, *possible* physiological substrates *Acta physiol scand* 1973 a. *88* 412—429

Barany E. H The liver like anion transport system in rabbit kidney uvea and choroid plexus. II Efficiency of acidic drugs and other anions as inhibitors *Acta physiol scand* 1973 b *88* 491—504

Heaton K. W *Bile salts in health and disease* Churchill Livingstone Edinburgh and London 19 2

Johnson J and N Kalant Bile composition in the pregnant rabbit. *Amer J dig Dis* 1972 *17* 1—6

Lack L. and I M Weiner Intestinal bile salt transport structure activity relationships and other properties *Amer J Physiol* 1966 *210* 1142—1152

Lundberg V Composite transport systems for iodipamide and iodohippurate out of the cerebrospinal fluid in the rat *Acta physiol scand* 1974 *9* 204—211

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From the Institute of Medical Pharmacology University of Uppsala Sweden

Composite Transport Systems for Iodipamide and Iodohippurate out of the Cerebrospinal Fluid in the Rat

By

VIVEKA LUNDBERG

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Abstract

LUNDBERG, VIVEKA *Composite transport systems for iodipamide and iodohippurate out of the cerebrospinal fluid in the rat* Acta physiol scand 1974 92 204—211

The mechanism for the removal of iodohippurate and iodipamide from the CSF in rats was studied by ventriculo-cisternal perfusion and by uptake experiments with choroid plexus *in vitro* In agreement with findings in other species excised plexus from the lateral ventricles accumulates ^{131}I o-iodohippurate and ^{125}I iodipamide and the process is at least partly saturable Ventriculo-cisternal perfusions were performed on pentobarbital anesthetized rats with a fluid containing ^{131}I o-iodohippurate ^{125}I iodipamide and ^{14}C inulin A considerable loss of iodohippurate and iodipamide but not of inulin from the CSF was observed during the perfusion When sodium hippurate and/or non labelled iodipamide were added in excess to the infusion fluid the loss of labelled iodohippurate and iodipamide was reduced In concentrations which reduced iodohippurate loss equally hippurate and iodipamide had different effects on iodipamide loss hippurate had less effect This indicates that iodohippurate and iodipamide are removed from the CSF partly by saturable and presumably active transport and that iodipamide is transported both by the same system as iodohippurate and also by a separate hippurate insensitive one Choroid plexus can be one locus of this transport

Saturable transport mechanisms removing various anions from the cerebrospinal fluid (CSF) have been studied by several workers using ventriculo cisternal perfusion (Pappenheimer Heisey and Jordan 1961 Davson Kleeman and Levin 1962, Pollay and Davson 1963 Bito Bradbury and Davson 1966 Cutler Robinson and Lorenzo 1968 Snodgrass *et al* 1969 Dixon Owens and Rall 1969 Cutler 1970 Lorenzo Hammerstad and Cutler 1970 Murray and Cutler 1970 Oldendorf *et al* 1971, Davson and Hollingsworth 1973 Snodgrass and Lorenzo 1973 Spector and Lorenzo 1973) In many cases these anions were also shown to be accumulated by choroid plexus *in vitro*

It has recently been found (Barany 1972 1973) that isolated choroid plexus as well as anterior uvea and slices of kidney cortex from a number of species incubated *in vitro* can concentrate not only o iodohippurate (Hippuran®) but also the cholangiographic agent iodipamide (Biligrafin®, a dicarboxylic acid) and that ac

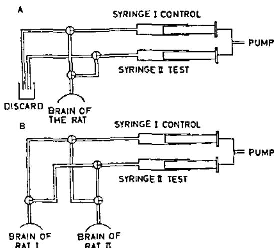


Fig 1 Diagram of arrangement for infusion of 2 different fluids one at a time into one of the lateral ventricles The length of the polyethylene tubings going from syringe to brain was 70 cm and internal diameter 0 5 mm A set up with one rat B set up with two rats

tive transport is the responsible mechanism Iodipamide is actively accumulated by at least two different systems one identical with the hippurate system and one which is separate The direction of transport of this latter is not clear

The purpose of the present investigation was to study whether the separate transport mechanism for iodipamide in the choroid plexus of the rat is outwards directed Uptake experiments with choroid plexus *in vitro* showed that the rat plexus has the separate iodipamide system and ventriculo-cisternal perfusion *in vivo* indicates that this system is outwards directed

Methods

In vitro experiments of iodohippurate and iodipamide uptake by excised choroid plexus from the lateral ventricles of the rat were performed as described by Barany (1972) The same solutions were used but the test substances had to be present in higher chemical concentrations because of the small amounts of tissue

Ventriculo cisternal perfusions were performed on female albino rats weighing 120—380 g The animals were anesthetized with pentobarbital 40 mg/kg intraperitoneally At the same time they were given methylatropine 1 mg/kg subcutaneously to reduce salivation and mucous secretion. The rats were placed on an electric heating pad. One of the lateral ventricles was cannuled for the inflow as described by de Balbian Verster *et al* (1971) For the outflow the atlanto-occipital membrane was exposed and a short pointed needle (0 6×25 mm) with the hub removed was connected to polyethylene tubing and inserted into the cisterna magna. The exit of the outflow tubing was set at 8 cm below the cisterna magna. The effluent was collected during successive 10 min periods in tared tubes and the amount was determined by weighing The inflow-cannula could be connected through polyethylene tubing and chemically inert valves (Hamilton no 3) to either one of two 5 ml syringes mounted on the same infusion pump (Fig 1 shows the different set ups schematically) One syringe delivers control fluid and the other one test fluid which is composed of control fluid and a putative inhibitor Approximately 30 s were required to switch from one syringe to the other during the experiment The infusion rate was approximately 15 µl/min The exact value was determined by calibrating the pump gravimetrically after each perfusion Injection of methylene blue as a marker was employed for checking the position of the inflow-cannulae at the termination of some of the experiments

TABLE I Uptake expressed as tissue/medium ratios after 20 min of iodohippurate and iodipamide by rat choroid plexus incubated in vitro The values which are corrected for unspecific uptake are means ± S E for the numbers of tissue pieces given in brackets

	Iodohippurate
Medium conc 2 μM iodohippurate	2 72 ± 0 29 (6)
20 μM iodohippurate + 20 μM iodipamide	0 97 ± 0 13 (6)
Unspecific uptake	0 51 ± 0 03 (4)
	Iodipamide
Medium conc 0 5–2 μM iodipamide	8 94 ± 1 46 (11)
20 μM iodohippurate + 20 μM iodipamide	4 16 ± 0 45 (6)
Unspecific uptake	0 68 ± 0 07 (4)
Medium conc 1 μM iodipamide	86 04 ± 5 03 (28)* dry weight

* is not corrected for unspecific uptake

The basal perfusion fluid had the following composition in mmol/l NaCl 128 KCl 3 0 NaH_2PO_4 0 48 $NaHCO_3$ 26 $CaCl_2$ 1 8 $MgSO_4$ 0 41 glucose 3 6 urea 3 3 (Fenstermacher 1972) In many experiments 1 ml of isotonic non radioactive NaI solution was added to 100 ml of the infusion fluid This is sufficient to saturate the I transport system in the choroid plexus (Becker 1961) It made no detectable difference The fluid was adjusted to pH 7 35 with 9 % CO_2 in N Because of the loss of some CO_2 through the tubing this represents a lower bound Iodipamide labelled with ^{125}I (Radiochemical Centre Amersham England) and iodohippurate labelled with ^{131}I (AB Atomenergi Studsvik Sweden) were added to the perfusion fluid to give a concentration of 2 μM of each isotope just prior to use ^{14}C labelled inulin (Amersham) was added to the perfusion fluid as an indicator of CSF secretion and bulk flow For the inhibition studies unlabelled iodipamide (Biligrafin forte® donated by Schering A G Berlin) and sodium hippurate (recrystallized Eastman Kodak or Schuchardt) were used either alone or together The concentration used of iodipamide was 2 mM and of sodium hippurate 10 mM The activity of ^{131}I o-iodohippurate ^{125}I iodipamide and ^{14}C inulin in the inflow medium and the outflow fractions were assayed in a Packard Tri Carb Spectrometer (2425 and 2099) 3 or 4 samples were taken from the inflow medium directly after the perfusion was terminated and 0 1 ml of each inflow and outflow sample were pipetted into glass scintillation vials to which 15 ml of a liquid scintillator (Bio solv BBS 3 (Beckman) 54 ml PPO 1 5 g dimethylPOPOP 0 09 g toluene 900 ml) were added The vials were preselected for equal empty weight this ensures approximately equal absorption of the gamma to be counted The vials were counted first in the gamma system and thereafter in the beta system of the spectrometer Corrections were made for decay background and spillover

Net transport N measured as the amount lost during the passage from lateral ventricle to cisterna magna was calculated by the following formula

$$N = C_{iT} \times v \quad (1 - c_{oT} \times c_{iI} / c_{iT} \times c_{oI}) \qquad (pmol/min)$$

where C_{iT} = concentration of ^{131}I o iodohippurate and ^{125}I iodipamide in the inflow (pmol/μl)
v = rate of inflow (μl/min)
c_{iT} and c_{oT} = activity of ^{131}I o-iodohippurate and ^{125}I iodipamide in the inflow and outflow respectively (cpm/0 1 ml)
c_{iI} and c_{oI} = activity of ^{14}C inulin in the inflow and outflow respectively (cpm/0 1 ml)
(c_o represents the mean of several 10 min sampling periods of effluent during steady state)

The secretion rate of cerebrospinal fluid V_f measured by dilution of the inulin was calculated by the following formula

$$V_f = v_i (c_{iI}/c_{oI} - 1) \quad (\mu l/min)$$

in which the symbols have the same meaning as described above

The secretion rate was also directly determined as the difference in weight between fluid infused and fluid effluxed per unit time

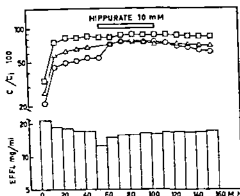


Fig 2a

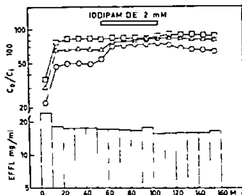


Fig 2b

Fig 2 Outflow concentration (c_o) of ^{131}I-o-iodohippurate ^{125}I iodipamide and ^{14}C-inulin expressed as percent of their inflow concentration (c_i) during ventriculo-cisternal perfusion 2 representative experiments a) 10 mM sodium hippurate during part of the experiment. b) 2 mM iodipamide during part of the experiment The histograms show the amount of effluent (EFFL) obtained during the collecting periods O ^{131}I-o-iodohippurate △ ^{125}I iodipamide □ ^{14}C-inulin

Results

Table I shows the results of the *in vitro* experiments Means of uptakes expressed as tissue/medium ratios found for different concentrations of ^{131}I-o-iodohippurate and ^{125}I iodipamide in the incubation medium are given The wet weights of choroid plexus were used for calculating the tissue/medium ratios except for the last group of tissues which were weighed dry The data are corrected for unspecific uptakes which were measured by incubating a few choroid plexus at 0° C It can be seen that iodohippurate and iodipamide are accumulated by the choroid plexus The means 3 23 and 9 62 for iodohippurate and iodipamide respectively are quite similar to those obtained by incubating isolated choroid plexus of rabbits (Barany 1972) An increase to 20 μM of the concentrations of both tracers in the medium resulted in reduced uptakes to 36 % for iodohippurate and to 47 % for iodipamide (tissue/medium ratios 0 97 and 4 16 respectively) This indicates that the accumulation process is at least partly saturable In view of this observation it was decided to study the transport mechanism *in vivo* by ventriculo cisternal perfusions

Fig 2a shows a representative experiment At zero time the perfusion was started by infusing a control fluid containing ^{131}I-o iodohippurate ^{125}I iodipamide and ^{14}C inulin and the effluent collected The concentration of the labelled substances in the effluent expressed as a percentage of their concentration in the infusion fluid is plotted semilogarithmically as a function of time After approximately 15 min of perfusion the concentration of the labelled substances in the effluent became reasonably steady The concentration ratios of inulin indicate the dilution of the infusion fluid by newly secreted CSF as inulin is considered to be transported by bulk flow

TABLE II Net transport (pmol/min) of iodohippurate and iodipamide from CSF during ventriculo-cisternal perfusion with and without inhibitor in the infusate Values are means ± S E for the numbers of animals *given in brackets* The percent inhibition of net transport caused by the inhibitor(s) is also given When percent inhibition has been calculated from the mean for each group it has been called total (Tot) When using the percent values of inhibition obtained *for each individual rat for* calculating the mean it has been called individual (Ind) The no inhibitor part (A) was always run first results from the post inhibitor phase *are not listed* The experiments either consisted of parts A+B A+C B+D or any one of A B C D singly 30 pmol/min of ^{131}I iodipamide and ^{131}I-o-iodohippurate *respectively were infused* The infusion rate was 15 µl/min The concentration of ^{14}C inulin in the infusate was approximately 30 000 cpm/0 1 ml

	A	B			C			D	
	No inhibition	Sodium hippurate 10 mM	Inhibition		Iodipamide 2 mM	Inhibition *		Sodium hippurate 10 mM+iodipamide 2 mM	Inhibition *
			Tot	Ind		Tot	Ind		Tot
^{131}I iodipamide	6 81 ±0 35 (15)	3 94 ±0 30 (14)	42 (14)	46 (5)	1 36 ±0 36 (10)	80 (10)	87 (6)	0 90 ±0 24 (11)	87 (11)
^{131}I o iodo-hippurate	13 59 ±1 18 (14)	3 61 ±0 39 (14)	73 (14)	69 (5)	3 61 ±0 62 (10)	73 (10)	77 (6)	1 63 ±0 40 (11)	88 (11)

only (Pollay and Davson 1963 Heisey Held and Pappenheimer 1962) From this first control period it can be noted that some of the infused ^{131}I iodipamide and ^{131}I-o-iodohippurate is lost during the passage from the lateral ventricle to the cisterna magna but it is difficult to determine whether this reflects diffusion and/or active transport from the CSF After a steady state had been held for 40 min the *infusion fluid* was changed to one containing 10 mM sodium hippurate in addition to the tracer substances The increase in concentration of ^{131}I iodipamide and ^{131}I-o iodohippurate in the effluent which resulted from this change indicates an inhibitory effect of sodium hippurate on the exit of ^{131}I iodipamide and ^{131}I o hippurate from the CSF This suggests that iodipamide and o iodohippurate are actively transported out of the CSF Following this test period of 50 min the control fluid was again infused and a slow decrease in the concentration of ^{131}I iodipamide and ^{131}I-o iodohippurate in the effluent was noted over the next 50 min However the concentration of ^{131}I iodipamide and ^{131}I o-iodohippurate in the effluent did not decrease during this time to the steady state level obtained during the initial control period. Thus the inhibitory effect of sodium hippurate was at least partly reversible It may well have been completely reversible but unsufficient time may have been allowed during the final period for complete recovery to occur The histogram at the lower part of Fig 2a represents the amount of effluent obtained during the collecting periods Although occasionally some variability in the outflow rate occurred, no significant change in the concentration ratio of ^{14}C inulin could be

observed This means that the sudden decrease in outflow rate which occurred *e g* after 50 min of perfusion in the experiment shown in Fig 2a was not due to a reduced CSF secretion rate but probably to an impediment in the outflow causing either a leakage at the cisternal needle or an increased volume of fluid in the ventricular space In any experiment the concentration of inulin in the effluent never varied by more than 5 % from the steady state mean In most experiments the variation was considerably less

Fig 2b shows the results of a corresponding experiment with excess of unlabelled iodipamide added instead of sodium hippurate It is observed that 2 mM iodipamide also had an inhibiting effect on the removal of both ^{125}I iodipamide and ^{131}I o-iodo-hippurate from the CSF the concentration of ^{125}I iodipamide and ^{131}I o iodo-hippurate in the effluent increased when unlabelled iodipamide was added to the infusion fluid

Table II shows means of net transport (pmol/min) values obtained from several such experiments with and without inhibitor in the infusate The net transport which is the amount of tracer lost from the CSF during its passage from lateral ventricle to cisterna magna includes both diffusion and active transport of the tracer The percent inhibition of net transport caused by 10 mM sodium hippurate and/or 2 mM iodipamide is also shown in the table When percent inhibition has been calculated from the mean for each group it has been called total When using the percent values of inhibition obtained for each individual rat for calculating the mean it has been called individual The net transport of ^{125}I iodipamide was found to be 6 81 pmol/min and of ^{131}I-o iodohippurate 13 59 pmol/min when 30 pmol/min of tracer substance were infused and no inhibitor was present Sodium hippurate in a concentration of 10 mM in the infusion fluid reduced the net transport of both ^{125}I iodipamide and ^{131}I-o iodohippurate significantly (by 42 % and 73 % resp) When the infusion fluid contained 2 mM iodipamide instead of 10 mM sodium hippurate a still greater reduction in the net transport of ^{125}I iodipamide was noticed while the reduction in the net transport of ^{131}I-o-iodohip purate was the same as with hippurate These findings show that the iodipamide transport system can be divided into one part that is sensitive to hippurate and therefore probably identical with the system transporting o iodohippurate (the hippurate system) and another part with very little affinity to hippurate (the hip-purate insensitive system) In the last group of experiments both 10 mM sodium hippurate and 2 mM iodipamide were simultaneously added to the infusion fluid No difference in the transport of ^{125}I iodipamide was noted between this group and the group perfused with only iodipamide as the inhibitor However the group per fused with both inhibitors did show a decreased transport of ^{131}I-o-iodohippurate compared to the group perfused with hippurate alone This may indicate that o iodohippurate is transported to a small extent by the iodipamide system

Measured by dilution of inulin mean ($\pm$S E) rate of CSF secretion was found to be 2 35 $\pm$ 0 13 μl/min (n = 34) This value is quite consistent with that found by Cserr (1965) 2 2 μl/min Measured by the difference between gravimetrically

determined inflow and outflow, CSF secretion rate was 2 08±0 09 µl/min (n = 30) This discrepancy is probably due to bulk absorption of the fluid after the passage from lateral ventricle to cisterna magna, and/or leakage at the cisternal needle, or low molecular impurities in the commercial inulin (Cohen 1969)

Discussion

There are 3 ways by which substances can be eliminated from the CSF active transport diffusion and bulk flow Ventriculo-cisternal perfusion in the pentobarbital anesthetized animal is a convenient technique that permits their separation and study even if pentobarbital has a moderate inhibitory effect on active transport from the CSF (Lorenzo Hammerstad and Cutler 1968) Inulin is eliminated from the CSF almost exclusively by bulk flow with a negligible amount leaving by diffusion across ventricular walls Therefore the dilution which occurs during the passage of inulin from the lateral ventricle to the cisterna magna is the result of newly secreted CSF The period to period variation in the concentration of inulin in the effluent seen in some experiments may have been due to difficulties in its determination by liquid scintillation counting (Bray 1970)

The results of the present *in vivo* study indicate that iodohippurate and iodipamide are eliminated from the CSF by means other than bulk flow as the recovery of infused iodohippurate and iodipamide in the effluent was significantly less than that of inulin The inhibition experiments showed that iodohippurate and iodipamide are removed from the CSF partly by saturable and presumably active tran port Iodipamide was found to be transported both by the hippurate system and a separate hippurate insensitive one The site of these transport systems cannot be determined from the results of the *in vivo* experiments Choroid plexus of the lateral ventricles is believed to be responsible for the transport largely because isolated plexus of the lateral ventricles were shown to accumulate iodohippurate and iodipamide Additional possibilities difficult to prove or rule out are the CSF brain and the brain blood interfaces

The specificity of the transport systems in choroid plexus have been investigated *in vitro* by incubating isolated choroid plexus with many different organic acids (Barany 1972 1973) However the physiological substrates of the hippurate insensitive iodipamide transport system are not known Maybe it transports bile acids and similar compounds cholic acid is transported out of the CSF in the rat and iodipamide is able to depress this transport (Lundberg unpublished)

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References

DEBALBIAN VERSTER F C A ROBINSON C A HENGEVELD and E S BUSH Freehand cerebroventricular injection technique for unanesthetized rats *Life Sci* 19 1 *10* 1305—1402

BARANY E H Inhibition by hippurate and probenecid of in vitro uptake of iodipamide and o-iodohippurate A composite uptake system for iodipamide in choroid plexus kidney cortex and anterior uvea of several species *Acta physiol scand* 1972 *86* 12—27

BARANY E H The liver like anion transport system in rabbit kidney uvea and choroid plexus I Selectivity of some inhibitors direction of transport possible physiological substrates *Acta physiol scand* 1973 *88* 412—429

BECKER B Cerebrospinal fluid iodide *Amer J Physiol* 1961 *201* 1149—1151

BITO L Z M W B BRADBURY and H DAVSON Factors effecting the distribution of iodide and bromide in the central nervous system *J Physiol* (Lond) 1966 *185* 323—354

BRAY G A Determination of radioactivity in aqueous samples In *The Current Status of Liquid Scintillation Counting* ed by E D Branscombe Grune and Stratton New York 1970 pp 170

COHEN S R A rapid sensitive semimicro gel filtration procedure for detecting and re moving low molecular weight fragments from [3H] — or [14C] — labeled inulin *Analyt Biochem* 1969 *31* 539—544

CSERR, H Potassium exchange between cerebrospinal fluid plasma and brain *Amer J Physiol* 1965 *209* 1219—1226

CUTLER R W P Transport of lysine from cerebrospinal fluid of the cat *J Neurochem* 1970 *17* 1017—1027

CUTLER R W P R J ROBINSON and A V LORENZO Cerebrospinal fluid transport of sulfate in the cat. *Amer J Physiol* 1968 *214* 448—454

DAVSON H and J R HOLLINGSWORTH Active transport of ^{131}I across the blood brain barrier *J Physiol* (*Lond*) *1973 233 327—347*

DAVSON H C R. KLEEMAN and E LEVIN Quantitative studies of the passage of different substances out of the cerebrospinal fluid *J Physiol* (Lond.) 1962 *161* 126—142

DIXON R L E S OWENS and D P RALL Evidence of active tran port of benzyl ^{14}C-penicill n from cerebrosp nal fluid to blood *J pharm Sci* 1969 *58* 1106—1109

FENSTERMACHER J D Ventriculocisternal perfus on as a technique for studying transport and metabolism within the brain *Res Meth Neurochem* 1972 *1* 165—178

HEISEY S R D HELD and J R PAPPENHEIMER, Bulk flow and diffusion in the cerebrospinal fluid system of the goat *Amer J Physiol* 1962 *203* 775—781

LORENZO A V J P HAMMERSTAD and R. W P CUTLER The effect of anaesthetic agents on the cerebrospinal fluid clearance of ^{3} S-sulphate and ^{1} I iodide *Biochem Pharmacol* 1968 *17* 1279—1283

LORENZO A V J P HAMMERSTAD and R. W P CUTLER Cerebrospinal fluid formation and absorption and transport of iodide and sulfate from the spinal subarachnoid space *J Neurol Sci* 1970 *10* 247—258

MURRAY J E and R W P CUTLER Transport of glycine from the cerebrospinal fluid *Arch Neurol* (Chic) 1970 *23* 23—31

OLDENDORF W H W B SISSON A C MEHTA and L TRECIOKAS Brain uptake of seleno-methionine Se 75 I Effects of elevated blood levels of methionine and phenylalanine *Arch Neu ol* (Chic) 1971 *24* 423—430

PAPPENHEIMER, J R S R HEISEY and E F JORDAN Active transport of Diodrast and sulphonphthalein from cerebrospinal flu d to blood *Amer J Physiol* 1961 *200* 1—10

POLLAY M and H DAVSON The passage of certain substances out of the cerebrospinal fluid *Brain* 1963 *86* 137—150

SNODGRAS S R and A V LORENZO Transport of GABA from the perfused ventricular system of the cat *J Neurochem* 1973 *20* 761—769

SNODGRASS S R R W P CUTLER E S KANG and A V LORENZO Transport of neutral amino acids from fel ne cerebrospinal flu d *Amer J Physiol* 1969 *217* 974—980

SPECTOR R and A V LORENZO The active transport of para aminosalicylic acid from the cerebrospinal fluid *J Pharmacol exp Ther* 1973 *185* 642—648

Acta physiol scand 1974 92 212—216

From the Chemical Techn Department, by Alco Helsinki Finland

Effects of Ethanol on Urine Sodium and Potassium Concentrations and Osmolality in Water-Loaded Rats

By

JAAKKO LINKOLA

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Abstract

LINKOLA, J *Effects of ethanol on urine sodium and potassium concentrations and osmolality in water loaded rats* Acta physiol scand 1974 92 212—216

The effects of ethanol on urine sodium potassium and osmolality were investigated in rats kept in a positive water balance produced by prior consecutive water loadings Ethanol loading was followed by rapid rises in urine output sodium concentration and osmolality and a somewhat slower rise in potassium concentration Presumably water loading led to an increased glomerular filtration rate which with the inhibition of ion reabsorption caused by ethanol was responsible for this rise

Ethanol has generally been found to reduce the urinary excretion of sodium and potassium (Nicholson and Taylor 1938 Rubini *et al* 1955 Ogata *et al* 1968 Kalbfleisch *et al* 1963 Klingman and Haag 1958) and to lower urine osmolality (Ogata *et al* 1968 Kleeman *et al* 1955 Roberts 1963) However some exceptions have been reported regarding the effects of ethanol on the excretion of Na and K Huang *et al* 1957 found the usual decrease after large amounts of alcohol but increased excretion after small doses Kleeman *et al* (1955) did not observe any changes in solute excretion when ethanol was given at the height of water diuresis According to Kruck and Krecke (1965) the excretion of sodium and potassium rises after ingestion of dilute ethanol solutions From these reports it was suggested that the hydration state might be a factor determining whether ethanol increases or decreases the excretion of sodium and potassium The present study was designed to test the possibility that a positive water balance produced by prior water loading would cause a rise rather than a fall in urine Na and K concentrations and osmolality during ethanol diuresis Kleeman *et al* (1955) and Kruck and Krecke (1965) made their experiments in the steady state phase of urine output but the present experiment was done during the phase in which water input exceeds urine output In this phase which is not stable, the glomerular filtration rate (GFR) rises (Chasis and Smith 1938 Daugharty *et al* 1972 Sonnenberg and Solomon 1969) and there is an increase in the passage of fluid and ions through the kidney

TABLE I Urine volumes [UV] concentrations of sodium [Na] and potassium [K] in urine and urine osmolality [Osm] in rats during the experiment Each figure represents the mean of 10 rats±S D P values for differences are beneath and between the figures

Time	[UV] ml/kg h		[Na] mmol/l		[K] mmol/l		[Osm] mOsm/kg	
	W	E	W	E	W	E	W	E
9 a m	11 8±4 4	13 2±7 0	17 9±10 1	18 6±8 2	8 3±4 5	9 0±3 8	216±157	232±109
10 a m	21 0±5 9	20 2±6 8						
11 a m	21 8±7 2	20 0±6 1	4 9±0 9	5 3±1 5	4 8±1 5	4 6±0 9	85±10	91±21
12 a m	25 6±1 9	50 6±9 6	3 7±1 2	10 7±5 8	3 7±0 4	4 5±[illegible] 4	80±4	119±26
	$p < 0.001$		$p < 0.005$		$p > 0.10$		$p < 0.001$	
1 p m	27 0±5 6	35 0±6 1						
	$p < 0.01$							
2 p m	16 3±2 4	10 2±3 4	4 4±1 2	14 7±4 0	3 7±0 6	10 9±[illegible] 7	88±5	216±36
	$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$	

tubules Hence the carrier system of the tubule wall is more strongly stimulated by the filtrate than in the steady state and the *effects of ethanol on ion transport are* probably more intense

Material and Methods

20 male Sprague Dawley rats weighing 414—500 g were used in this experiment. They had been given standard ASTRA EWOS (Sodertalje Sweden) rat food and tap water freely until the beginning of the experiment A positive water balance was produced by giving to each rat 30 ml per kg body weight of distilled water at hourly intervals *from 8 to 10 in the* morning (a m) with a stomach tube At 11 a.m 10 rats again received distilled water while the other ten received 30 ml per kg b wt of 10 % v/v ethanol in distilled water administered by stomach loading At noon all rats were given distilled water again After each intubation the rats were put into metabolic *cages for urine collection Care was taken that no urine* was lost when the rats were out of the cages Urine samples were measured hourly and collected at 9 and 11 a m at noon and at 2 p m Urine sodium and potassium were analyzed with a flame photometer (JOUAN Paris France) and osmolality with an osmometer (FISKE Uxbridge Massachusetts USA) Statistical analyses were performed with Student's t test

Results

Urine output Urine output increased progressively during water loading but did not reach the input level of 30 ml/kg h in 5 h (Table I) A positive water balance was thus ensured After ethanol loading there was a phase of diuresis for about 2 h in which *urine output increased further The difference between* the ethanol group and the control group was significant in samples taken from 11 to 12 a m ($p < 0.001$) and from noon to 1 p m ($p < 0.01$) The diuresis phase was followed by a clear antidiuresis phase in which the ethanol group excreted significantly ($p < 0.001$) less urine

Sodium and potassium concentrations of urine The repeated water loading from 8 to 12 a m decreased both the sodium and the potassium concentration of the urine in control rats (Table I) After alcohol gavage at 11 a m there was a rapid increase in the sodium concentration and a somewhat slower increase in the potassium concentration of the urine 11—12 a m , $p < 0\ 005$ for sodium and $p > 0\ 10$ for potassium 1—2 p m , $p < 0\ 001$ for both ions The increase in Na concentration was simultaneous with the rise in urine output, but elevated concentrations of sodium and potassium continued into the beginning of the antidiuresis phase Thus during the diuresis phase between 11 a m and 1 p m the rats treated with ethanol lost more sodium and potassium than the controls

Osmolality of urine Water loading decreased urine osmolality markedly from 8 to 12 a m in the control group (Table I) After ethanol loading at 11 a m urine osmolality increased significantly as compared with the corresponding values for the control group 11—12 a m , $p < 0\ 001$ and 1—2 p m $p < 0\ 001$

Discussion

The present results show that in rats in a positive water balance ethanol raises the output of urine and simultaneously increases the urinary sodium and potassium concentrations This is in contrast to the commonly accepted belief that ethanol decreases the urinary output of monovalent ions (Nicholson and Taylor 1938 Rubini *et al* 1955 Ogata *et al* 1968 Kalbfleisch *et al* 1963 Klingman and Haag 1958) In those studies a positive water balance was not ensured nor was the glomerular filtration rate increased intentionally The results are however in agreement with the findings of Kruck and Krecke (1965), although these authors suggested that the natriuretic effect in their experiment was probably not due to ethanol but to hydration In their experiment the effects of ethanol were hard to distinguish from those of hydration and this led to the design of the present experiment Kleeman *et al* (1955) used prehydration in their experiment but like Kruck and Krecke (1965) they kept urine flow steady in human beings during the experiment

Active transport is known to be the mechanism mainly responsible of sodium reabsorption in the kidney tubules and this process is stimulated by aldosterone in the distal tubules (Edelman *et al* 1963 Keele and Neil 1971) The excretion of potassium depends on active reabsorption in the proximal tubules (Bloomer *et al* 1963 Malnic *et al* 1966) and collecting ducts (Giebisch and Windhager 1964) and distal tubular secretion (Malnic *et al* 1966 Berliner 1961, Malnic and Giebisch 1972) which is strongly determined by the transtubular potential gradient (Giebisch and Windhager 1964 Malnic *et al* 1966) There is evidence that alcohol inhibits active Na transport (Israel and Kalant 1963 Israel Jacard and Kalant 1965) and aldosterone function (Fabre *et al* 1969) By so doing it may decrease the reabsorption of ions and thus increase their excretion Ethanol has been suggested to enhance reabsorption of sodium and potassium by acting directly on the

kidney tubules by some unknown mechanism but in that context the water balance was not taken into account There is no evidence that active Na transport is dependent on the hydration state and if water loading had any effects on aldosterone function this would be seen in the results for the control group

Ethanol increases the excretion of inorganic phosphate (Markkanen and Nanto 1966) and the concentration of sodium and excretion of potassium in the distal tubules is affected by the presence of large anions (Giebisch and Windhager 1964 Giebisch and Malnic 1965 Giebisch *et al* 1966) When ethanol decreases the excretion of Na and K the GFR does not change (Rubini *et al* 1955) Hence the reabsorptive mechanisms of the tubule walls which interact with large anions may possibly operate in different ways when GFR is steady and when it is rising producing an increase in sodium and potassium excretion in the latter case Kruck and Krecke (1965) and Kleeman *et al* (1955) found no increase in GFR during the experiment and attributed their results to the positive water balance and ethanol alone It is possible that these results could be somehow explained on the basis of the possible changes in the transtubular potential gradient which would also affect ion reabsorption (Giebisch and Windhager 1964 Malnic *et al* 1966) Many factors can raise urine osmolality These include elevated sodium and potassium concentrations and increased excretion of inorganic phosphate (Markkanen and Nanto 1966) The presence of ethanol and lactate in the urine may have the same effect

The present results do not fully explain the mechanisms by which sodium and potassium are excreted after ethanol loading But is is evident that water loading increases GFR and this with the inhibition of ion reabsorption caused by ethanol may at least partly account for the results This study lends support to the view that in ethanol intoxication ion balance may be differently affected by different levels of hydration

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References

BERLINER R W Renal mechanisms for potassium excretion *Harvey Lect* Ser 55 1961 141—172

BLOOMER H A F C RECTOR and D W SELDIN The mechanism of potassium reabsorption in the proximal tubules of the rat *J clin Invest* 1963 *42* 277—285

CHASL H and H W SMITH The excretion of urea in normal man and in subjects with glomerulonephritis *J clin Invest* 1938 *17* 347—358

DAUGHARTY T M I F UEKI D P NICHOLAS and B M BRENNER Comparative renal effects of isoncotic and colloid free volume expansion in the rat *Amer J Physiol* 1972 222 225—235

EDELMAN I S R BOGOROCH and G A PORTER On the mechanism of action of aldosterone on sodium transport Role of protein synthesis *P oc Nat Acad Sci* 1963 *50* 1169—1177

FABRE L F JR R W FARMER H W DAVIS J CROSS and G FARRELL Ethanol inhibition of the aldosterone response to hemorrhage *Fed Proc* 1969 28 No 2 635

GIEBISCH G and G MALNIC Effects of ionic substitutions on distal transtubular potential differences of a rat kidney *Fed Proc* 1965 *24* 570

GIEBISCH G and E E WINDHAGER Renal tubular transfer of sodium chloride and potassium *Am J Med* 1964 *35* 643—669

GIEBISCH G G MALNIC R M KLOSE and E E WINDHAGER Effect of ionic substitutions on distal potential differences in rat kidney *Amer J Physiol* 1966 *211* 560—568

HUANG K C P K KNOEFEL L SHIHOMURA and N KING BUREN Some effects of alcohol on water and electrolytes in the dog *Arch int Pharmacodyn* 1957 *109* 90—98

ISRAEL Y and H KALANT Effect of ethanol on the transport of sodium in frog skin *Nature* (Lond) 1963 *200* 476—478

ISRAEL-JACARD Y and H KALANT Effect of ethanol on electrolyte transport and electrogenesis in animal tissues *J cell comp Physiol* 1965 *65* 127—132

KALBFLEISCH J M R D LINDEMAN H E GINN and W O SMITH Effects of ethanol administration on urinary excretion of magnesium and other electrolytes in alcoholic and normal subjects *J clin Invest* 1963 *42* 1471—1475

KEELE C A and E NEIL Samson Wright's Applied Physiology *Oxford University Press* London New York Toronto 1971

KLEEMAN C R M E RUBINI E LAMDIN and F H EPSTEIN Studies on alcohol diuresis II The evaluation of ethyl alcohol as an inhibitor of the neurohypophysis *J clin Invest* 1955 *34* 448—455

KLINGMAN G I and H B HAAG Studies on severe alcohol intoxication in dogs I Blood and urinary changes in lethal intoxication *Quart J Stud Alcohol* 1958 *19* 203—225

KRUCK F and H J KRECKE The renal sodium excretion during oral hydration in man *Nephron* 1965 2 321—333

MALNIC G and G GIEBISCH Some electrical properties of distal tubular epithelium in the rat *Amer J Physiol* 1972 223 797—808

MALNIC G R M KLOSE and G GIEBISCH Micropuncture study of distal tubular potassium and sodium transport in rat nephron *Amer J Physiol* 1966 *211* 529—547

MALNIC G R M KLOSE and G GIEBISCH Microperfusion study of distal tubular potassium and sodium transfer in rat kidney *Amer J Physiol* 1966 *211* 548—559

MARKKANEN T and V NANTO The effect of ethanol infusion on the calcium phosphorus balance in man *Experientia* (Basel) 1966 22 753—754

NICHOLSON W M and H M TAYLOR The effect of alcohol on the water and electrolyte balance in man *J clin Invest* 1938 *17* 279—285

OGATA M J H MENDELSON and N K MELLO Electrolytes and osmolality in alcoholics during experimentally induced intoxication *Psychosom Med* 1968 *30* 463—488

ROBERTS K E Mechanism of dehydration following alcohol ingestion *Arch int Med* 1963 *112* 154—157

RUBINI E C R KLEEMAN and E LAMDIN Studies on alcohol diuresis I The effect of ethyl alcohol ingestion on water electrolyte and acid base metabolism *J clin Invest* 1955 *34* 439—447

SONNENBERG H and S SOLOMON Mechanism of natriuresis following intravascular and extracellular volume expansion *Canad J Physiol Pharmacol* 1969 *47* 153—159

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From the Department of Physiology University of Lund Sweden

Motor and Secretory Effects of Autonomic Nerves and Drugs in the Rat Submaxillary Gland

By

ANDERS THULIN

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Abstract

THULIN A *Motor and secretory effects of autonomic nerves and drugs in the rat submaxillary gland* Acta physiol scand 1974 *92* 217—223

The effect of autonomic nerves and drugs on duct pressure and salivary flow was investigated in rat submaxillary gland The results showed that it is possible to separate motor responses probably exerted by activation of myoepithelial cells from secretion It is concluded that both divisions of the autonomic nervous system evoke motor responses and that the sympathetic motor effect is mediated via α receptors

Histochemical and electron microscopical investigations have demonstrated that myoepithelial cells are present in the submaxillary gland of the rat (Leeson 1956 Scott and Pease 1959 Shear 1964 Tamarin 1965 Harrop and Mackay 1968) Axons partly bared of their Schwann cell investment have been observed in relation ship to these cells (Bogart 1970 Bogart *et al* 1973) No experiments on the function of the myoepithelial cells of the rat submaxillary gland have been reported In the present investigation the effect of stimulation of the autonomic nerves and of drugs on the pressure in the duct system and on the salivary flow of this gland was studied It is known that not only parasympathetic but also sympathetic nerve stimulation evokes a flow of saliva from the submaxillary gland of the rat (Ohlin 1965 Yoshida *et al* 1967) and the effect of the sympathetic nerve is mediated both by α and β adrenoceptors (Emmelin *et al* 1965)

Methods

40 male rats weighing 280—5?0 g of a Sprague Dawley and a Wistar strain were used They were anesthetized with chloralose (100 mg/kg) given through a cannula in a femoral vein after induction with ether Additional doses of the anesthetic were given when required The right or the left submaxillary duct was exposed and cannulated close to the mouth with a fine glass cannula. In 15 rats the chorda lingual nerve or the sympathetic trunk was exposed and stimulated from a Grass S4 stimulator with supramaximal strength (4—8 [V]) and with a duration of ? ms using single shocks or different frequencies during 1—4 min A bipolar

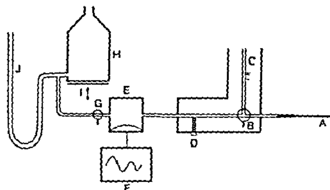


Fig 1 Pressure and volume recording system A glass cannula inserted in the submaxillary duct B three way stopcock C glass capillary to study secretion D micrometer screw for calibration E displacement transducer F polygraph G two-way stopcock H pressure bottle I table movable in vertical direction J water manometer

electrode was used The following drugs were injected intravenously acetylcholine methacholine atropine adrenaline noradrenaline phenylephrine isoprenaline dihydroergotamine propanolol and D (—) N isopropyl p-nitrophenylethanolamine (INPEA)

Pressure recordings The glass cannula inserted in the submaxillary duct was connected to a displacement transducer (Fig 1) by which the pressure changes in the duct system could be recorded Sometimes the saliva caused obstruction in the duct system. To overcome this the pressure in the duct was increased to 20—30 cm H_2O during 30 s before each recordings This was done by connecting the recording system with a pressure bottle (Fig 1) The effects of nerve stimulations and drugs were studied at pressure levels of 10—20 cm H_2O The smallest volume displacement which could be recorded by this system was 0 007 μl

Volume recordings The volume recordings were made by observing the meniscus in the fine glass capillary (Fig 1) using a dissecting microscope at × 10 magnification A volume displacement of 0 015 μl could be detected by this method When no stimulus was applied the level of the meniscus usually was steady 3—5 cm above the gland When the meniscus moved upward and did not return to the initial level this was attributed to secretion A dose of a drug or a frequency of nerve stimulation was considered to be subsecretory if it did not cause any movement of the meniscus or if the meniscus quickly returned to the initial level as further control such stimuli were repeated several times every 1—2 min to ascertain that no addition of fluid occurred in the glass capillary In 5 expts there was a slight constant flow of saliva and the secretory threshold to drugs and nerve stimulation could not be estimated A similar flow in occasional animals has been noticed by Emmelin *et al* (1965)

Results

Parasympathetic stimulation

A single shock applied to the chorda lingual nerve always raised the pressure in the closed system The latency between the application of the stimulus and the response was less than one second and the maximal rise varied between 1 and 4 5 cm H_2O Fig 2 shows pressure responses to single shocks and to different frequencies of chorda lingual stimulation

In the open system a single shock did not cause any sign of secretion This was studied in 8 rats In these rats shocks were then applied 10—20 times at intervals of 1 min In 7 of the rats no secretion could be detected in this way only in one there was a scanty addition of fluid and this occurred when 10 shocks had been given The frequency of stimulation had to be increased to 0 1 Hz in 5 and to 0 2 Hz in 2 of the rats before there was any discernible secretion

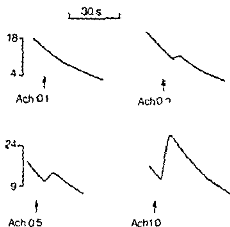


Fig 3 Pressure recordings to increasing doses of acetylcholine Numbers indicate doses in μg/kg Calibration cm H_2O

Parasympathomimetic drugs

Acetylcholine 0.02—0.2 μg/kg raised the duct pressure (Table I) The pressure rise increased with increasing doses Fig 3 shows the pressure responses to different doses of acetylcholine The threshold dose to evoke salivary secretion with acetylcholine was 0.5—1.0 μg/kg Similarly the threshold doses of methacholine to evoke pressure rise were lower than those for secretion (Table I)

In experiments on dogs a secretory effect of parasympathomimetic drugs often manifested itself in the closed system as a secondary rise in the pressure record

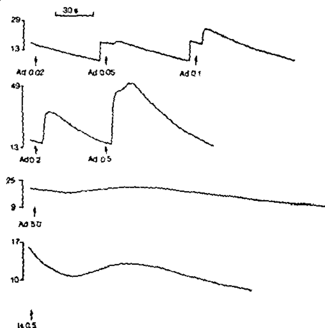


Fig 4 The upper two recordings show the pressure rises to increasing doses of adrenaline The lower recordings show the pressure rises to adrenaline 5.0 μg/kg after dihydroergotamine was given and to isoprenaline 0.5 μg/kg Calibration cm H_2O

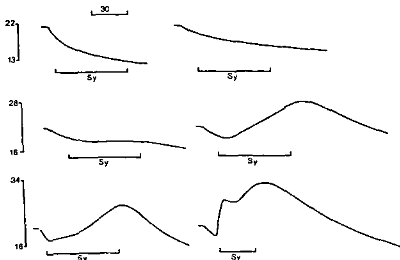


Fig 5 Pressure recordings to sympathetic stimulation Upper records 0 1 Hz and 0 2 Hz Middle records 0 5 Hz and 1 0 Hz Lower records 2 0 Hz and 5 0 Hz. Sy = sympathetic stimulation Calibration cm H_2O

(Emmelin *et al* 1969 a) such an effect was not observed in the present experiments on rats

Atropine 0 1—0 2 mg/kg abolished the pressure and secretory responses elicited by acetylcholine and methacholine Dihydroergotamine and propranolol did not diminish the effects of these drugs

Sympathomimetic drugs

Adrenaline noradrenaline and phenylephrine raised the pressure in the duct in doses far below those needed for secretion (Table I) With increased doses of the drugs the pressure responses became larger and steeper When doses above the secretory threshold were used the initial steep pressure rise was followed by a more gradual secondary rise (Fig 4) The pressure rise to subsecretory doses and the initial pressure response to doses above the secretory threshold were not affected by propanolol (1 mg/kg) or INPEA (5 mg/kg) but were abolished by dihydroergotamine (0 5—1 mg/kg) The secretion and the secondary pressure response following injection of phenylephrine were likewise unchanged after propranolol but were abolished by dihydroergotamine However these effects of adrenaline and noradrenaline were reduced by propranolol and were also diminished but not abolished by dihydroergotamine the α blocking drug caused a larger reduction of the responses than propranolol and when both blocking drugs were given no effects were obtained

Isoprenaline also caused a pressure rise and a flow of saliva but the threshold doses to obtain these effects were about the same (Table I) The pressure response resembled that described above as a secondary phase after a relatively long latency

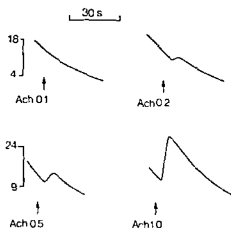


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Parasympathomimetic drugs
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In experiments on dogs a secretory effect of parasympathomimetic drugs often manifested itself in the closed system as a secondary rise in the pressure record

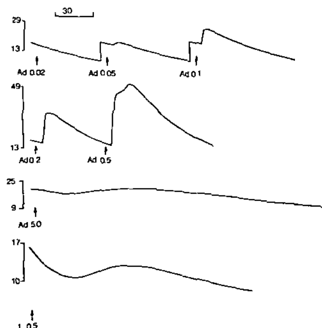


Fig 4 The upper two recordings show the pressure rises to increasing doses of adrenaline The lower recordings show the pressure rises to adrenaline 5 0 μg/kg after dihydroergotamine was given and to isoprenaline 0 5 μg/kg Calibration cm H_2O

latency between the activation by these drugs of the myoepithelial and secretory cells in rats

The effects of dihydroergotamine propranolol and INPEA indicate that secretion induced by sympathomimetic drugs is mediated via α and β receptors while the motor response is mediated only via α receptors

In these experiments the pressure rise to single chorda lingual shocks is probably an effect exerted by contraction of the myoepithelial cells The observation that a single shock to the chorda lingual nerve did not evoke secretion (with one exception) while the injection of fluid which mimicked the pressure response to a single chorda lingual shock could be observed in the open system supports the suggestion that the pressure effect is not a result exclusively of secretion

From the experiments with sympathomimetic drugs it was concluded that the threshold for activation of myoepithelial cells is lower than that of the secretory cells When the sympathetic nerve was stimulated signs of myoepithelial contractions were obtained only with frequencies which also caused secretion This might indicate that the spatial arrangement of the nerve is such that the secretory cells are much easier reached by the sympathetic transmitter than the myoepithelial cells

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References

BOGART B J Fine structural localization of cholinesterase activity in the rat submandibular gland *J Histochem Cytochem* 1970 *18* 730—739

BOGART B J and C L DE LEMOS Adrenergic innervation of the rat submandibular and parotid glands An electron microscopic autoradiographic study of the uptake of tritiated norepinephrine *Anat Rec* 1973 *177* 219—224

DARKE A C and L H SMAJE Myoepithelial cell activation in the submaxillary salivary gland *J Physiol* (Lond.) 1971 *219* 89—102

EMMELIN N J HOLMBERG and P OHLIN Receptors for catecholamines in the submaxillary glands of rats *Brit J Pharmacol* 1965 *25* 134—138

EMMELIN N P OHLIN and A THULIN The pharmacology of salivary myoepithelial cells in dogs *Brit J Pharmacol* 1969 a *37* 666—679

EMMELIN N J R GARRETT and P OHLIN Motor nerves of salivary myoepithelial cells in dogs *J Physiol* (Lond) 1969 b *200* 539—546

HARROP T J and B MACKAY Electron microscopic observations on myoepithelial cells and secretory nerves in rat salivary glands *J Canad dent Ass* 1968 *34* 480—488

LEESON C R Localization of alkaline phosphatase in the submaxillary gland of the rat *Nature* (Lond) 1956 *178* 311—312

OHLIN P Isoprenaline as a secretory agent in salivary glands *Acta Univ Lund* 1964 Section II No 17 3—8

OHLIN P Secretory responses of innervated and denervated submaxillary glands of rats *Acta Univ Lund* 1965 Section II No 23 3—12

SCOTT B L and D C PEASE Electron microscopy of the salivary and lacrimal glands of the rat *Amer J Anat* 1959 *104* 115—140

SHEAR M Histochemical localization of alkaline phosphatase and adenosine triphosphatase in the myoepithelial cells of rat salivary glands *Nature* (Lond) 1964 *203* 770

TAMARIN A Myoepithelium of the rat submaxillary gland *J Ultrastruct Res* 1966 *16* 320—338

YOSHIDA Y R L SPRECHER C A SCHNEYER and L H SCHNEYER Role of β receptors in sympathetic regulation of electrolytes in the rat submaxillary saliva *Proc Soc exp Biol* (N Y) 1967 *126* 912—916

Acta physiol scand 1974 92 224—237

From the Department of Physiology University of Goteborg Sweden

Effects of Gonadotrophins, Dibutyryl Cyclic AMP and Prostaglandin E on Amino Acid Transport and Glycolysis in Isolated Rat Corpora Lutea

By

HANS HERLITZ

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Abstract

HERLITZ H *Effects of gonadotrophins dibutyryl cyclic AMP and prostaglandin E_2 on amino acid transport and glycolysis in isolated rat corpora lutea* Acta physiol scand 1974 *92* 224—237

Corpora lutea (CL) were isolated by free hand dissection from ovaries of 34—39 day-old Sprague Dawley rats injected with 10 IU Pregnant Mare s Serum gonadotrophin (PMSG) when 30 days old leading to ovulation in the morning of day 33 Injection of luteinizing hormone (LH) to rats 2 h before removal of the ovaries stimulated the *in vitro* uptake of the non utilizable amino acids α aminoisobutyric acid (AIB-^{14}C) and 1 amino-cyclopentane carboxylic acid (cycloleucine-^{3}H) by 3-day-old CL during a subsequent incubation in Krebs bicarbonate buffer Prolactin and FSH were without effects. Addition of LH directly to the incubation medium did not stimulate amino acid uptake of the isolated CL while addition of dibutyryl cyclic AMP (dbcAMP) enhanced this uptake Glycolysis was estimated by determination of lactic acid accumulation in the incubation medium When LH was injected to the animals 2 h before sacrifice lactic acid production of 3-day-old CL was stimulated during a subsequent incubation period Addition of LH *in vitro* caused a slight increase in the rate of glycolysis and dbcAMP and prostaglandin E (PGE$_2$) also exhibited stimulatory effects on lactic acid production by the incubated CL In a few experiments rats with 7-day-old CL were given injections of LH While amino acid uptake was not stimulated in these old CL LH did enhance the rate of glycolysis in these CL

It is known that prolactin and luteinizing hormone (LH) serve as important regulatory factors for normal development function and regression of the corpus luteum (CL) of the rat (Short 1967) Our knowledge concerning the mechanism of action of these two gonadotrophins in their effects on CL is however still limited Most studies within this field have concentrated on the biosynthetic pathway for steroids and the effects of gonadotrophins on steroidogenesis and steroid secretion (*e g* Savard *et al* 1965) There are however some reports concerning effects of gonadotrophins on aspects of the cellular metabolism other than those involved directly in the steroidogenesis For example Armstrong and Greep (1962) found that the administration of LH 4 h prior to sacrifice increased the uptake of glucose

and the production of lactic acid by slices of heavily luteinized ovaries during subsequent *in vitro* incubation Their studies were confirmed and extended by Channing and Villee (1966) and by Flint and Denton (1969) Stansfield and Robinson (1965) reported a decrease in total glycogen and an increase in phosphorylase activity in CL of rats injected with LH Armstrong (1968) has suggested that the increased rate of glycolysis in the CL after LH stimulation is secondary to the steroidogenic effect of this hormone

Very few studies have been reported concerning hormonal effects on protein synthesis and protein metabolism in the CL Demers *et al* (1973) have recently reported that prolactin enhanced the rate of incorporation of amino acids into CL protein when CL from pseudopregnant rats were cultured for 24—72 h The prepubertal rat ovary has been more extensively studied in this respect (*e g* Ahren and Kostyo 1963 Ahren *et al* 1967) and it has been found that both FSH and LH have acute stimulatory effects on amino acid uptake and amino acid incorporation into protein when the hormones are injected to the rats *in vivo* and the prepubertal ovaries studied *in vitro* (Ahren and Kostyo 1963 Ahren *et al* 1967 Nilsson and Selstam 1974) It was therefore considered of interest to make similar studies on isolated CL with the hope that this type of experiments would give some information on the relationship between LH and prolactin in their luteotrophic action Since both cyclic AMP (cAMP) and prostaglandins (PGs) have been suggested to be involved in the mechanism of action of gonadotrophins the effects of these substances on the isolated CL were also studied

The first generation of CL from immature rats injected with a low dose of PMSG were used in order to have a well characterized and standardized preparation Two non utilizable amino acids α aminoisobutyric acid (AIB-^{14}C) and 1 aminocyclopentane carboxylic acid (cycloleucine ^{3}H) were used to study amino acid uptake Phenylalanine ^{3}H was used to study the rate of incorporation of amino acids into CL protein CL glycolysis *in vitro* was determined by measuring lactic acid production during the incubation period

Material and methods

Animals Female Sprague Dawley rats from Anticimex Ltd Stockholm Sweden were used They were kept in rooms with constant temperature (24—26 C) and relative humidity (50—55 %) and exposed to controlled illumination of 14 h daily starting at 5 a.m The rats were fed a standard pellet diet and water *ad libitum* 10 IU PMSG was given s c at 8—10 a.m to prepubertal rats on day 30 In a previous paper (Fuxe *et al* 1972) it was found that this PMSG dose gave a physiological number of ova at ovulation It is well known that a single injection of PMSG to immature rats can produce ovulation but that the effect is dependent upon endogenous release of LH from an intact hypophyseal pituitary system (*e g* Cole 1936 Zarrow and Quinn 1963) It has been shown in previous experiments that injection of nembutal before 2 a.m. on day 32 inhibits the ovulation and recent measurements of LH in plasma have revealed an endogenous LH release in the afternoon of day 32 (Hillensjo Barnea, Nilsson Herlitz and Ahren to be published) Ovulation occurs early in the morning of day 33 with formation of 10—20 CL In most animals these CL remain for 12—13 days at which time follicular growth and a new ovulation are seen CL were isolated from 35 and 39-day-old rats designated as 3 and 7-day-old CL (see Fig 1) Occasionally the number of ova in the tubal ampullae was less than the number of CL isolated in both ovaries

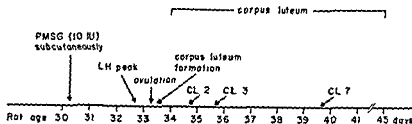


Fig 1 Time schedule for the PMSG model CL-2 CL 3 and CL-7 represent 2 3 and 7-day old cropora lutea (CL) respectively

The most likely explanation to this finding is that luteinization in some cases took place with the oocyte captured within the follicle This phenomenon has been discussed for the luteinized ovary (Villee *et al* 1969)

Rats injected with hormones intraperitoneally or intravenously in the tail were given a light ether anesthesia during the injection period

Hormones The hypophyseal hormones used were supplied by the Endocrinology Study Section of the National Institutes of Health Bethesda Md USA The designations and activities of these hormones as well as the stated contaminations with gonadotrophins (within brackets) were as follows Bovine LH NIH LH B8 (1.03 LH S1 units/mg less than 0.05 FSH S1 units/mg) ovine FSH NIH FSH S9 (1.13 FSH S1 units/mg less than 0.01 LH S1 units/mg) bovine prolactin NIH P S3 (15 prolactin IU/mg less than 0.009 FSH S1 units/mg less than 0.0009 LH S1 units/mg) Human chorionic gonadotrophin (HCG activity = 5300 IU/mg) was a gift from AB Leo Helsingborg Sweden and PMSG (Pregnant Mare's Serum gonadotrophin) was Gestyl® from N V Organon Oss The Netherlands

Chemicals The labelled substances were obtained from NEN (New England Nuclear) Boston USA The substances were used with the following specific activities and molarities α aminoisobutyric acid 1 ^{14}C (AIB ^{14}C) 1.0 μCi/μmol 0.1 mM L-phenylalanine-^{3}H 1980 μCi/μmol 0.01 mM sucrose 6 6 ^{3}H 79.2 μCi/μmol 0.02 mM 1 aminocyclopentane 1 carboxylic acid carboxyl ^{14}C (cycloleucine ^{14}C) 1.0 μCi/μmol 0.1 mM N^{6} ^{2}' O-dibutyryl 3' 5' AMP was purchased from Sigma Chemical Co St Louis Mo USA PGE_2 was made available by the courtesy of Ono Pharmaceutical Co Ltd Osaka Japan

Removal and incubation of corpora lutea The rats were killed by cervical fracture the ovaries rapidly removed and dissected free Under a stereomicroscope CL were excised by means of stainless steel needles and transferred to ice cold Krebs bicarbonate buffer pH 7.3—7.4 until sufficient material was accumulated for the incubation Five CL each weighing 1—1.5 mg were then blotted on a moist filter paper rapidly weighed on a torsion balance and placed in 10 ml Erlenmeyer flasks containing 1 ml Krebs bicarbonate buffer with 5.5 mM glucose and half the usual calcium concentration The flasks were gassed with 95 % O_2—5 % CO_2 and sealed with tightly fitting rubber stoppers The CL were incubated for various times at 37 °C with continuous shaking Following incubation the flasks were placed on ice and the media withdrawn immediately The CL were removed from the flasks rinsed free of adhering incubation medium by a rapid wash in cold buffer and blotted on filter paper before further anlayses (see below)

Measurement of lactic acid production Lactic acid was determined by an enzymatic method described by Lundholm *et al* (1963) The lactic acid production was calculated from the amount of lactic acid found in the medium at the end of the incubation period While this represents only part of the total production experiments on isolated prepubertal ovaries have shown that changes in the total production under various experimental conditions were reflected in the lactic acid accumulation in the medium Lactic acid production is expressed as μg produced per 100 mg of tissue during the total incubation period (2 h)

Measurement of total and extracellular tissue water Total tissue water was determined by drying CL to constant weight in a 100 °C oven The values are expressed as per cent of the wet tissue weight (= ml water in 100 g tissue) The total water content of 3-day-old CL was found to be 81.8 ± 0.7 % For determining the extracellular water the CL were incubated with 0.02 mM sucrose ^{3}H in the medium After the incubation the CL were homogenized in 1 ml 10 % trichloroacetic acid (TCA) and the percentage distribution of ^{3}H in the CL was determined by counting the radioactivity of appropriate aliquots of the protein free TCA

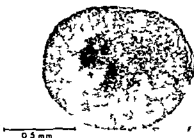


Fig 2 Photomicrograph of a paraffin embedded histological section of a 3-day old corpus luteum (CL) stained with van Gieson

0 5 mm

extracts and of the media The radioactivity was assayed in a Packard Tri Carb liquid scintillation spectrometer Model 3375 The sucrose space of the 3-day-old CL was found to be 31 3±0 7 % after 15 min of incubation No difference in the space was noted with increasing incubation time up to 240 min indicating that the sucrose ^3H equilibrated with the extracellular tissue fluid within 15 min and that it did not penetrate into the intracellular water

Measurements of the intracellular accumulation of AIB ^{14}C and cycloleucine ^{14}C After the incubation the CL were homogenized in 1 ml 10 % TCA and the radioactivity of the total tissue water was determined by counting 0 2 ml aliquots of the protein free TCA extracts diluted with 7 ml Instagel (Packard) The amount of the model amino acid in the extracellular water was calculated assuming that the concentration in the extracellular water was the same as in the incubation medium and this amount was subtracted from the total amount of AIB-^{14}C or cycloleucine ^{14}C present in the tissue, probably giving a measure of the concentration of this amino acid in the intracellular compartment The degree of quenching was tested by automatic external standardization in each sample and was found to be the same for the tissue extracts and their respective media It was therefore not necessary to correct the counting data for quenching prior to the calculations of extracellular spaces and distribution ratios The intracellular accumulation of the substances is expressed as the distribution ratio of radioactivity between the intra and extracellular compartments (cpm/ml intracellular water cpm/ml medium) Double labelled samples were counted in the same spectrometer with settings which gave a 10—15 % overlap of ^{14}C radioactivity in the ^3H channel and a 1 % overlap of ^3H activity in the ^{14}C channel

Measurements of the accumulation of phenylalanine ^3H After incubation with the labelled amino acid the CL were homogenized in 10 % TCA and the radioactivity determined as described above This amino acid is probably metabolized to some extent in the CL cells and it is therefore likely that some of the radioactivity in the tissue extracts did not represent the original amino acid but various metabolites from it This has not been analysed in the present experiments It is thus important to point out that the distribution ratio for this amino acid is the ratio between the total intracellular radioactivity and the radioactivity in the incubation medium (= cpm/ml intracellular water cpm/ml incubation medium)

Measurements of incorporation of phenylalanine ^3H into protein After incubation the CL were homogenized in 1 ml 10 % TCA The precipitate was spun down and washed once in 10 % TCA The precipitate was heated for 10 min at 90 C in 3 ml of 10 % TCA in the presence of excess of non labelled amino acid After centrifugation the precipitate was washed twice with 3 ml of a mixture of ethanol ether chloroform (2 2 1) The purified protein was dissolved in 0 5 ml NaOH and was heated in a boiling water bath for 5 min Total protein was determined according to Lowry *et al* (1951) using bovine serum albumin as a standard (Sigma) For determining the radioactivity of the protein fractions the washed protein precipitate as dissolved in 0 5 ml 1N NaOH and 50 µl was taken from this solution for measurement of radioactivity Liquid scintillation counting was carried out with 10 ml of a solution consisting of 90 % PPO (2 5-diphenyloxazole) and 10 % Bis MSB[p-Bis (0 methyl styryl) benzene] (Permablend TM III Packard) in toluene (5 5 g/1000 ml) in a Packard liquid scintillation spectrometer with a counting efficiency of 32—34 % for ^3H Duplicate samples were always counted DPM (disintegration per minute) values for individual samples were calculated and incorporation of radioactivity into protein is expressed as DPM/µg protein

Histological sections In some of the experiments paraffin sections were prepared from CL of different age These sections (5 µm thick) were stained with van Gieson and eosin and studied under the microscope From Fig 2 it can be seen that it is possible to dissect the CL

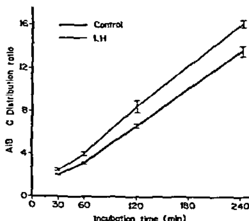


Fig 3

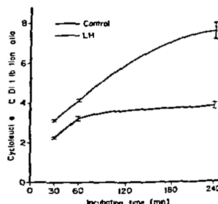


Fig 4

Fig 3 Accumulation of AIB by isolated 3-day-old corpora lutea (CL) LH was injected i.p (10 μg/rat) 2 h prior to incubation CL were incubated in Krebs bicarbonate buffer pH 7 4 containing 0 1 mM AIB-^{14}C and 5 5 mM glucose Each point represents the mean of 7 observations and S.E is indicated by a vertical line. The difference between the results from control and LH injected rats were significant already after 30 min incubation. ($p < 0\,05$)

Fig 4 Accumulation of cycloleucine by isolated 3-day-old corpora lutea (CL) LH was injected i.p (10 μg/rat) 2 h prior to incubation. CL were incubated in Krebs bicarbonate buffer pH 7 4 containing 0 1 mM cycloleucine ^{14}C and 5 5 mM glucose. Each point is the mean of 5 observations and S.E is indicated by a vertical line. The difference between the results from control and LH injected rats was significant after 30 min incubation ($p < 0\,05$)

almost entirely free from surrounding tissue In some cases, however minute follicles have been left at the surface of CL.

Statistical procedure Mean values and standard error of the mean are given. The significance of differences was calculated by analysis of variance (with one criterion of classification followed by the Student Newman Keuls multiple range test (Woolf 1968) A p-value of 0 05 or less was considered significant.

Results

Observations on 3 day-old corpora lutea

Amino acid transport and protein biosynthesis Fig 3 and 4 show that the time courses of the cellular uptake of the two non utilizable amino acids in isolated 3-day-old CL did not differ from those reported from other tissues such as the prepubertal rat ovary the intracellular concentration of AIB continued to increase linearly for the whole incubation period (4 h) cycloleucine initially was accumulated more rapidly than AIB but the distribution ratio came to a steady state after approximately 60 min. Fig 3 also shows that CL from rats injected with LH 2 h before sacrifice showed significantly higher AIB distribution ratios at all incubation periods studied. Distribution ratios after 2 h of incubation were therefore used in further experiments as measure of the rate of uptake of this model amino acid. Fig 4 illustrates that CL from the LH injected rats displayed a more rapid initial rate of uptake of cycloleucine and the distribution ratio after 4 h of incubation was 100 % higher than in the control CL.

TABLE I Effects of gonadotrophic hormones *in vivo* on amino acid transport and protein biosynthesis in isolated 3 day-old corpora lutea (CL) *in vitro*

Hormone dose (μg/rat)	AIB-¹⁴C distribution ratio	Phenylalanine ³H distribution ratio	Phenylalanine ³H incorporation DPM/μg prot
Exp I			
Saline	6 88±0 29		
LH 1	8 18±0 38		
LH 10	8 37±0 39		
LH 100	8 10±0 17*		
FSH 10	7.25±0 21		
FSH 100	7 69±0 22		
Exp II			
Saline	4 47±0 29	2 49±0 07	180± 7
LH 10	6 35±0 20	3 97±0 11*	179±12
FSH 10	4 61±0 19	2 51±0 38	162± 8
Exp III			
Saline	3 95±0 22	1 79±0 07	154± 3
LH 10	5 76±0 20*	3 08±0 07**	164± 7
Prolactin 10	3 85±0 05	2 03±0 07	150± 8
Exp IV			
Saline	4 60±0 14	2 20±0 07	208± 5
LH 10	6 16±0 17*	2 99±0 06**	247± 3*
Prolactin 10	4 59±0 12	2 11±0 05	235± 9
HCG 10	6 12±0 17*	3 40±0 16	259±12*

4 different expts are presented in which the hormones were administrated i v or i p under light ether anesthes a 2 h before sacrifice In expt I and II the hormones were injected i p while i v *injection was used in expts III and IV No difference has been found between these two manners* of administration Five CL were incubated in each flask for 2 h in Krebs bicarbonate buffer containing 0 1 mM AIB ¹⁴C and 5 5 mM glucose In the 3 last expts 0 01 mM phenylalanine ³H was also present in the incubation medium Each group unless otherwise indicated numbers the mean of 5 observations±S E Statistically significant effects are indicated

* $p < 0.05$ ** $p < 0.01$

Table I shows 4 different experiments in which various gonadotrophic hormones were administered i v or i p to rats 2 h before removal of the ovaries followed by a 2 h incubation in presence of AIB ¹⁴C and phenylalanine ³H No difference in effects was found between an i v or an i p administration of the hormone It can be seen that 1 μg LH per rat was enough to stimulate the AIB transport and that no further increase was seen with 10 or 100 μg In addition LH also elicited a marked increase in the phenylalanine ³H accumulation by CL of this age In one experiment (IV) CL from the LH injected rats showed a slightly increased incorporation of phenylalanine ³H into protein but in the other experiments LH had no such effect FSH and prolactin influenced neither the AIB transport nor the accumulation of phenylalanine A few preliminary experiments were performed to test the hypothesis that prolactin might augment the response to LH LH and prolactin were given i v together and the effect on the AIB uptake during a subsequent incubation was compared to that obtained with LH alone The results are seen in Table II The AIB distribution ratios were in all three experiments higher

TABLE II Augmentation by prolactin of the LH effect *in vivo* on AIB transport in 3 day old corpora lutea (CL)

Hormone dose (μg/rat)	AIB ¹⁴C distribution ratio	Significance of the difference from LH alone
Exp I		
Saline	6 21 ± 0 27	
LH 10	7 55 ± 0 11	
Prolactin 10	6 34 ± 0 14	
LH 10 + prolactin 10	10 29 ± 0 48	$p < 0\ 01$
Exp II		
Saline	4 C4 ± 0 17	
LH 10	6 63 ± 0 44	
Prolactin 10	—	
LH 10 + prolactin 10	7 62 ± 0 32	$0\ 05 < p < 0\ 11$
Exp III		
Saline	5 71 ± 0 24	
LH 1	6 0(± 0 23	
Prolactin 10	5 46 ± 0 10	
LH 1 + prolactin 10	6 85 ± 0 34	$0\ 05 < p < 0\ 09$

The hormones were injected i v under light ether anesthesia 2 h before sacrifice Five CL were incubated in each flask for 2 h in Krebs bicarbonate buffer containing 0 1 mM AIB ¹⁴C and 5 5 mM glucose Each group represents the mean of 5 observations ± S E

when prolactin was injected together with LH but the augmentation of the LH effects was statistically significant only in one experiment

CL were also incubated in presence of gonadotrophins *in vitro* No hormonal effects were seen on amino acid uptake or amino acid incorporation into protein In another series of experiments incubations were carried out in presence of dbcAMP or PGs The results are summarized in Table III With dbcAMP a clear stimulation of the amino acid transport was seen while PGF_2 showed no influence None of these substances could affect protein synthesis

TABLE III Effects of dbcAMP and PGF on intracellular accumulation of AIB ¹⁴C and phenylalanine ³H and on the incorporation of phenylalanine ³H into protein by isolated 3-day old corpora lutea (CL)

	AIB ¹⁴C distribution ratio	Phenylalanine ³H distribution ratio	Phenylalanine ³H incorporation DPM/μg prot
Control	5 52 ± 0 20	2 53 ± 0 11	195 ± 15
dbcAMP 1 mM	8 36 ± 0 30 *	4 10 ± 0 18**	212 ± 27
dbcAMP 5 mM	9 50 ± 0 81**	5 40 ± 0 28**	23(± 8
PGF_2 1 μg/ml	6 43 ± 0 31	3 10 ± 0 13	215 ± 18
PGF_2 10 μg/ml	6 30 ± 0 41	3 18 ± 0 09	219 ± 19

5 CL were incubated in each flask for 2 h in Krebs bicarbonate buffer containing 0 1 mM ... 0 01 mM phenylalanine ³H and glucose 5 5 mM The values are given as mean ± ... represents the mean of 5 observations ± S E Statistically significant effects are ...
* $p < 0\ 01$

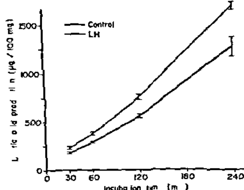


Fig 4

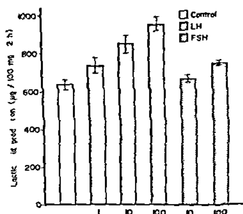


Fig 5

Fig 5 Lactic acid production by isolated 3-day-old corpora lutea (CL) LH was injected i.p. (10 μg/rat 2 h before incubation CL were incubated for 30—240 min in Krebs bicarbonate buffer containing 5 5 mM glucose Each point represents the mean of 6 observations and S E is indicated by a vertical line The effect of LH is significant at each point studied

Fig 6 Effects of different doses of LH and FSH *in vivo* on lactic acid production by isolated 3-day-old corpora lutea (CL) LH or FSH were injected 2 h before removal of the ovaries Amounts given are indicated in the figure (μg/rat) CL were incubated for 2 h in Krebs bicarbonate buffer containing 5 5 mM glucose Means ± S E are given There are 5 observations in each group Statistically significant effects are indicated x $p < 0.05$ xx $p < 0.01$

TABLE IV Effects of LH and HCG *in vitro* on lactic acid production by isolated 2—3-day-old corpora lutea (CL)

Group (μg/ml medium)	Lactic acid production μg/100 mg × 2 h		Group (μg/ml medium)	Lactic acid production μg/100 mg × 2 h	
Exp I			Exp III		
Control	574±21	(5)	Control	685±29	(4)
LH 10	703±73	(5)	LH 10	902±54*	(4)
LH 100	786±41	(5)	LH 100	912±67*	(4)
			HCG 10	876±19*	(4)
Exp II					
Control	550±32	(5)			
LH 10	616±33	(5)			
HCG 20	619±26	(5)			

In the first 2 expts 3-day-old CL were used while CL from day 2 were studied in the last expt 5 CL were incubated for 2 h in Krebs bicarbonate buffer containing 5 5 mM glucose in absence and presence of LH or HCG Lactic acid accumulation in the medium was determined at the end of the incubation period Values are mean ± S E Number of observations are given in parentheses Statistically significant effects are indicated

* $p < 0.05$

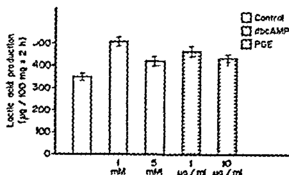


Fig 7 Effects of dbcAMP and PGE on lactic acid production by 3-day-old corpora lutea (CL) CL were incubated for 2 h in Krebs bicarbonate buffer containing 5 5 mM glucose and dbcAMP or PGE₂ Means ± S E are given There are 5 observations in each group Statistically significant effects are indicated x $p<0\,05$ xx $p<0\,01$

Lactic acid production The rate of glycolysis was estimated by determining lactic acid accumulation in the incubation medium Fig 5 shows a time course study of lactic acid production by 3-day-old CL from control rats and rats injected with LH The hormone was injected (10 µg/rat) i p 2 h before sacrifice It can be seen that this type of *in vivo* administration increased the rate of *in vitro* glycolysis Fig 6 shows the dose response relationship for LH *in vivo* on lactate production *in vitro* A slight effect was seen with 1 µg of the hormone with further increase with 10 and 100 µg Fig 6 also shows that a significant effect was not obtained with FSH

When 2—3-day-old CL were incubated *in vitro* in presence of LH or HCG a slight increase in glycolysis was also obtained The mean values were always higher for the LH stimulated groups but the effect was not statistically significant in all experiments (see Table IV) Incubations were also performed with dbcAMP and PGE Fig 7 illustrates the effects of these compounds on lactic acid production. dbcAMP enhanced glycolysis in a concentration of 1 mM while 5 mM displayed a smaller stimulatory effect PGE_2 (1 µg/ml) also significantly increased lacate formation with a slightly smaller effect with 10 µg/ml

TABLE V Influence of LH on intracellular accumulation of AIB-C and the lactic acid production by 7-day-old corpora lutea (CL)

	AIB-C distribution rat	Lactic acid production µg/100 mg / 2 h
Exp I		
Saline	4 33 ± 0 19	3[illegible]3 ± 14
LH	4 10 ± 0 20	477 ± [illegible]*
Exp II		
Saline	[illegible]17 ± 0 27	27[illegible] ± 1[illegible]
LH	[illegible]00 ± 0 31	4[illegible]6 ± 42

5 CL were incubated in each flask for 2 h in Krebs bicarbonate buffer containing 0 1 mM AIB-C and 5 5 mM glucose 10 µg of LH dissolved in 0 9 saline was injected 2 h before removal of the ovaries Controls were injected with saline Values are given as mean ± S E There are 5 observations in each group Statistically significant effects are indicated

* $p < 0\,05$

Observations on 7 day old corpora lutea

Table V shows that injection of LH 2 before removal of the ovaries did not enhance AIB uptake in these older CL while LH still stimulated CL glycolysis

Discussion

A newly formed CL of the rat has in principle the potential to develop into one of three different conditions 1) the CL of the cycling animal showing functional luteolysis after 4—5 days as judged by increased activity of the enzyme 20 α hydroxy steroid dehydrogenase (20 α OH SDH) 2) the CL of the pseudopregnant rat with a life span of 11—12 days or 3) the CL of pregnancy which displays luetolysis after 20—21 days (Wiest *et al* 1968) Hashimoto and Wiest (1969) have described an experimental CL model using the immature rat injected with 4 IU PMSG in the morning of day 26 with ovulation and CL formation in the morning of day 29 The life span of this CL was short showing signs of functional luteolysis after 4—5 days In our PMSG model the CL persisted for a much longer time corresponding to that of pseudopregnancy Using this model increased activity of 20 α OH SDH was first detected in 10 day old CL (Lamprecht Herlitz and Ahren to be published) The reason for this marked increase in the duration of the CL in our experimental model compared to that of Hashimoto and Wiest (1968) is unknown There are however clear differences in the experimental conditions as we have used older rats and higher doses of PMSG

Earlier studies have shown that the effects of gonadotrophins on CL vary markedly with the stage of development and function of this ovarian structure Both prolactin and LH can act as luteotrophic factors and stimulate progesterone secretion under certain conditions (Astwood 1941 Armstrong *et al* 1964 Takayama and Greenwald 1973) while the same hormones under other experimental conditions can produce luteolysis (Rothchild 1965 Malven *et al* 1966) Synergistic as well as antagonistic effects between these two gonadotrophins have also been reported under various experimental conditions (Browning *et al* 1962 Hauser and Malven 1971) It is therefore important to define very precisely the type of CL used in experiments concerned with the regulatory control by gonadotrophins on the metabolism of CL In the present study 3 and 7 day-old CL were studied The 3 day-old CL is a young but fully developed CL as judged from weight and protein content Moreover Basset (1943) demonstrated that the luteinization is completed and the vascular architecture developed at this time While the 7-day old CL is approaching the end of its life span the lack of 20 α OH SDH activity (Lamprecht *et al* 1974) indicates that it has not begun to undergo luteolysis

Under these conditions it was found that LH stimulated amino acid uptake in the young CL but not in the older one Amino acid uptake was studied with the two non utilizable amino acids AIB and cycloleucine which have been shown to have quite different kinetics in their intracellular accumulation during *in vitro* incubation of other tissues (Akedo and Christensen 1962 Segal *et al* 1966 Ahren *et al* 1967) We have demonstrated a similar difference in uptake kinetics between

AIB and cycloleucine with isolated CL The increase in intracellular concentration of AIB was linear for a very long period (at least up to 4 h), while cycloleucine which was initially taken up more rapidly came to a steady state distribution after 30—60 min It has been shown for other tissues (Christensen and Jones 1962 Ahren *et al* 1967) that the kinetics for cycloleucine uptake simulate that of most normal amino acids more closely than do the kinetics of AIB uptake The fact that LH stimulated the rate of uptake of both these model amino acids as well as the steady state distribution of cycloleucine makes it very likely that LH also stimulates the uptake of normal amino acids in the 3 day old CL

A hormonal stimulation of amino acid uptake is usually an expression of a growth stimulating (anabolic) effect within its target cells and is usually concomitant with an increased rate of incorportaion of amino acids into protein LH did however not stimulate incorporation of phenylalanine into protein of the 3 day old CL The very slight increase in incorporation in one experiment (Table I) can easily be explained as a consequence of an increase in specific activity of the intracellular phenylalanine due to increased uptake of the labelled amino acid The lack of effect of LH on amino acid incorporation makes it unlikely that the LH stimulated amino acid uptake represents an anabolic effect It is possible that CL cells can use amino acids for generation of energy and that an increased uptake of amino acids is one of the means by which enough energy is formed to cope with an increased steroidogenesis Whether CL cells contain enzymes for catabolism of amino acids is not known but it is interesting that an alanine cycle found in some extrahepatic tissues (*e g* skeletal muscle) provides a means by which amino acids can be metabolized without release of the toxic metabolite amonia (Mallette 1969) In this cycle alanine is formed by amino transfer to pyruvate from several amino acids It would be of interest to investigate whether such an alanine cycle exists in CL cells

The fact that prolactin did not in itself stimulate amino acid uptake but had a clear tendency to augment the effect of LH (Table II) is interesting in relation to earlier reports about synergistic effects between LH and prolactin on CL Armstrong (1969) has thus found that prolactin facilitates the ability of LH to stimulate the turnover of cholesterol in isolated PMSG induced CL Browning *et al* (1962) have also given morphological evidences that LH and prolactin have synergistic actions as luteotrophic factors in the mouse

LH did not stimulate amino acid transport in 7 day old CL Measurements of progesterone secretion have not yet been done in our CL model but it is interesting that other investigators (*e g* Yoshinaga *et al* 1967) have reported that the stimulatory effect of LH on progesterone secretion is most pronounced in young CL When progesterone secretion into the ovarian vein was measured in adult cycling rats Yoshinaga *et al* (1967) found a pronounced stimulatory effect of LH during early diestrous while a less marked stimulation was seen in late diestrous Even more interesting is that they did not find any stimulatory effect of LH on day 5 of pseudopregnancy

Addition of dbcAMP to 3-day-old CL stimulated amino acid uptake. Similar effects of dbcAMP have been reported in other endocrine glands, *e.g.* the thyroid (Burke *et al.* 1971) and the prepubertal rat ovary (Ahren and Hamberger 1969). It is however doubtful that cAMP is involved as a mediator in the above mentioned *in vivo* effect of LH on amino acid transport since *in vitro* addition of LH of 3 day-old CL markedly increased cAMP formation (Herlitz *et al.* 1974) without any stimulation of amino acid uptake.

CL glycolysis was first investigated by Armstrong *et al.* (1962) who incubated slices of heavily luteinized ovaries from immature rats injected with PMSG and HCG according to the schedule described by Parlow (1958) for use in the ovarian ascorbic depletion test for LH. Armstrong found that while injection of LH 30—120 min before incubation stimulated the rate of glycolysis in the isolated CL slices, it had no effect when added directly to the incubation medium. Armstrong (1968) has suggested that the LH effect on CL glycolysis is not directly linked to the increased progesterone secretion produced by this gonadotrophin but plays a role in replenishment of the cholesterol stores depleted by LH. The use of heavily luteinized ovaries of PMSG+HCG injected rats has been critisized (Guillet and Rennels 1964) since the metabolism and function of this tissue might be different from that of a normal CL. In the present study it was demonstrated that LH also stimulated glycolysis in our PMSG model. While this effect was seen both on 3 and 7 day-old CL, one clear difference was noted in relation to Armstrong's results. In the present experiments LH stimulated glycolysis both when it was injected to the rats before extirpation of the ovaries and when it was added directly to the incubation medium. The reason for this difference is not known but it is not unlikely that CL cells in various stages of development have different capacity to respond to LH *in vitro*. This suggestion is strengthened by the recent finding that the stimulatory effect of LH on *in vitro* cAMP formation in isolated CL decreased dramatically with the age of the CL (Herlitz *et al.* 1974).

Both cAMP and PGs have been suggested as possible mediators of the effects of LH on the ovary. Both substances have been found to mimic the action of LH on glycolysis when added to isolated prepubertal ovaries (Perklev and Ahren 1971, Herlitz *et al.* 1974). *The present study shows that dbcAMP and PGE_2 can mimic* the *in vitro* effect of LH on CL glycolysis and it has been found in other experiments that PGE_2 also increased cAMP formation in the isolated CL. It therefore seems possbile that the glycolytic effect of LH in CL is mediated as in the prepubertal rat ovary (Herlitz *et al.* 1974) at least in part by cAMP. Less evidence exists in favour of the theory (Kuehl *et al.* 1970) that PGs should be obligatory mediators in the action of LH (for disc. see Ahren *et al.* 1974).

In conclusion it should be pointed out that this experimental CL model seems to be very suitable for studies intended to investigate the influence of various stimuli on CL function, *i.e.* both factors promoting CL growth and progesterone secretion and factors favouring CL regression.

The continuous interest of professor Kurt Ahrén in this work is gratefully acknowledged Thanks are also due to Dr Gunnar Selstam for valuable suggestions Expert technical assistance was given by Mrs Anita Sjogren The author also wishes to thank the Endocrinology Study Section of NIH AB Leo and N V Organon for the generous supply of hormones and Ono Pharmaceutical Co for the gift of prostaglandins

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References

AHRÉN K and L HAMBERGER Effects of cyclic 3 5 AMP on ovarian amino acid transport *Acta physiol scand* 1969 *Suppl 330* 75

AHRÉN K and J L KOSTYO Acute effects of pituitary gonadotrophins on the metabolism of isolated rat ovaries *Endocrinology* 1963 *73* 81—91

AHRÉN K L HAMBERGER and M HARTFORD Further studies on the effect of follicle stimulating hormone on amino acid transport in the isolated rat ovary *Acta physiol scand* 1967 *71* 211—223

AHRÉN K H HERLITZ L NILSSON T PERKLEV S ROSBERG and G SELSTAM Gonadotrophins and cyclic AMP in various compartments of the rat ovary In *Hormones and Polypeptides Vol 7* Eds Li C H and N R Moudgal Academic Press New York 1974 In press

AKEDO H and H N CHRISTENSEN Nature of insulin action on amino acid uptake by the isolated diaphragm *J biol Chem* 1962 *237* 118—122

ARMSTRONG D T Gonadotropins ovarian metabolism and steroid biosynthesis *Recent Progr Horm Res* 1968 *24* 255—319

ARMSTRONG D T Luteotrophic roles of prolactin and luteinizing hormone in the rat In *Proceedings of the third internat congress of endocrinology* Ed Gual C Excerpta Medica Foundation Amsterdam 1969 89—96

ARMSTRONG D T and D O GREEP Effect of gonadotrophic hormones on glucose metabolism by luteinized rat ovaries *Endocrinology* 1962 *70* 701—710

ARMSTRONG D T J O BRIEN and R O GREEP Effects of luteinizing hormone on progestin biosynthesis in the luteinized rat ovary *Endocrinology* 1964 *75* 488—500

ASTWOOD E B The regulation of corpus luteum function by hypophysial luteotrophin *Endocrinology* 1941 *28* 309—320

BASSETT D L The changes in the vascular pattern of the ovary of the albino rat during the estrous cycle *Amer J Anat* 1943 *73* 251—291

BURKE G K KOWALSKI and S YU Effects of 3 5 cyclic nucleotides on membrane transport in isolated thyroid cells *Acta endocr* (Kbh) 1971 *68* 183—195

BROWNING H C G A LARKE and W D WHITE Action of purified gonadotrophins on corpora lutea in the cyclic mouse *Proc Soc exp Biol* (N Y) 1962 *111* 686—690

CHANNING C P and C A VILLEE Luteinizing hormone Effects on uptake and metabolism of hexoses by luteinized rat ovaries *Biochim biophys Acta* (Amst) 1966 *115* 205—218

CHRISTENSEN H N and J C JONES Amino acid transport models Renal resorption and resistance to metabolic attack *J biol Chem* 1962 *237* 1203—1206

COLE H H On the biological properties of mare gonadotropic hormone *Amer J Anat* 1936 *59* 299—331

DEMERS L M H R BEHRMAN and R O GREEP Effects of prostaglandins and gonadotrophins on luteal prostaglandin and steroid biosynthesis In *Advances in the Biosciences Vol 9* International Conference on Prostaglandins Ed Bergstrom S Pergamon Press/Vieweg Braunschweig 1973 701—707

FLINT A P F and R M DENTON Glucose metabolism in the superovulated rat ovary *in vitro* *Biochem J* 1969 *112* 243—254

FUXE K T HOKFELT C D SUNDSTEDT K AHRÉN and L HAMBERGER Amine turnover changes in the tubero-infudibular dopamine (DA) neurons in immature rats injected with PMS *Neuroendocrinology* 1972 *10* 287—300

GUILLET G G and E G RENNELS The experimental production of deciduomata in immature rats *Texas Rep Biol Med* 1964 *22* 78—93

HASHIMOTO I and W G WIEST Correlation of the secretion of ovarian steroids with function of a single generation of corpora lutea in the immature rat *Endocrinology* 1969 *84* 873—885

HAUSLER C L and P V MALVEN Interaction between prolactin [illegible] neurohypophysial hormones and hysterectomy on luteal function in rats *Biol Reprod* 1971 *5* 262—269

HERLITZ H L. HAMBERGER and K AHREN Comparison between effects of cyclic AMP dibutyryl cyclic AMP and luteinizing hormone on ovarian glycolysis Acta physiol scand 1974 92 181—194
HERLITZ H L HAMBERGER S ROSBERG and K AHREN Cyclic AMP in isolated corpora lutea of the rat Influence of gonadotrophins and prostaglandins *Acta endocr* (Kbh) 1974 In press
KUEHL J F A J L HUMES J TORNOFF V J CIRILLO and E A HAM Prostaglandin receptor site Evidence for an essential role in the action of luteinizing hormone *Science* 1970 *169* 883—886
LAMPRECHT S H HERLITZ and K AHREN Effect of prostaglandin $F_{2\alpha}$ on first generation corpora lutea in gonadotrophin treated immature rats Induction of 20 α hydroxysteroid dehydrogenase To be published
LOWRY O H N J ROSEBROUGH A L FARR and R J RANDALL Protein measurement with the Folin phenol reagent *J biol Chem* 1951 *193* 265—275
LUNDHOLM L E MOHME LUNDHOLM and N VAMOS Lactic acid assay with L(+) lactic acid dehydrogenase from rabbit muscle *Acta physiol scand* 1963 *58* 243—249
MALLETTE L E J H EXTON and C R PARK Control of gluconeogenesis from amino acids in the perfused rat liver *J biol Chem* 1969 *244* 5713—5723
MALVEN P V and C H SAWYER A luteolytic action of prolactin in hypophysectomized rats *Endocrinology* 1966 *79* 268—274
NILSSON L and G SELSTAM Effects of LH and HCG on the metabolism of the prepubertal rat ovary Stimulation of amino acid transport protein synthesis and glycolysis *Acta endocr* (Kbh) 1974 In press
PARLOW A F A rapid bioassay method for LH and factors stimulating LH secretion *Fed Proc* 1958 *17* 402
PERKLEV T and K. AHREN Effect of prostaglandins LH and polyphloretin phosphate on the lactic acid production of the prepubertal rat ovary *Life Scie* 1971 *10* 1387—1393
ROTHCHILD I The corpus luteum hypophysis relationship The luteolytic effect of luteinizing hormone (LH) in the rat *Acta endocr* (Kbh) 1965 *49* 107—119
SAVARD K J M MARSH and B F RICE Gonadotrophins and ovarian steroidogenesis *Recent Progr Horm Res* 1965 *21* 285—365
SEGAL S H ROTH A BLAIR and D BERTOLI, Accumulation of amino acids by thyroid gland slices *Endocrinology* 1966 *79* 675—680
SHORT R V Reproduction *Ann Rev Physiol* 1967 *29* 373—400
STANSFIELD D A and J W ROBINSON Glycogen and phosphorylase in bovine and rat corpora lutea and the effect of luteinizing hormone. *Endocrinology* 1965 *76* 390—395
TAKAYAMA M and G S GREENWALD Hormonal requirements for the maintenance of luteal function in hypophysectomized pseudopregnant rats *J Endocr* 1973 *56* 421—429
VILLEE C A C P CHANNING B ECKSTEIN and V SULOVIC Effects of gonadotrophins on the metabolism of luteinized and follicular ovarian tissues In *The Gonads* Ed McKerns K V Appleton Century Crofts New York 1969 291
WIEST W G W R. K KIDWELL and K. BALOGH JR. Progesterone catabolism in the rat ovary A regulatory mechanism for progestational potency during pregnancy *Endocrinology* 1968 *82* 844—859
WOOLF C M *Principles of biometry* D van Nostrand Co Inc USA 1968
YOSHINAGA K S A GRIEVES and R V SHORT Steroidogenic effects of luteinizing hormone and prolactin on the rat ovary in vivo *J Endocr* 1967 *38* 423—430
ZARROW M X and D L QUINN Superovulation in the immature rat following treatment with PMS alone and inhibition of PMS induced ovulation *J Endocr* 1963 *26* 181—188

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References

Ahrén K. and I Hamberger, Effects of cyclic 3 5 AMP on ovarian amino acid transport. *Acta physiol scand* 1969 *Suppl 330* 75

Ahrén K. and J L Kostyo Acute effects of pituitary gonadotrophins on the metabolism of isolated rat ovaries *Endocrinology* 1963 *73* 81—91

Ahrén K. L Hamberger and M Hartford Further studies on the effect of follicle stimulating hormone on amino acid transport in the isolated rat ovary *Acta physiol scand* 1967 *71* 211—223

Ahrén K H Herlitz L Nilsson T Perklev S Rosberg and G Selstam Gonadotrophins and cyclic AMP in various compartments of the rat ovary In *Hormones and Polypeptides Vol 7* Eds Li C H and N R Moudgal Academic Press New York 1974 In press

Akedo H. and H N Christensen Nature of insulin action on amino acid uptake by the isolated diaphragm *J biol Chem* 1962 *237* 118—122

Armstrong D T Gonadotropins ovarian metabolism and steroid biosynthesis *Recent Progr Horm Res* 1968 *24* 255—319

Armstrong D T Luteotrophic roles of prolactin and luteinizing hormone in the rat. In *Proceedings of the third internat congress of endocrinology* Ed Gual C Excerpta Medica Foundation Amsterdam 1969 89—96

Armstrong D T and D O Greep Effect of gonadotrophic hormones on glucose metabolism by luteinized rat ovaries *Endocrinology* 1962 *70* 701—710

Armstrong D T J O'Brien and R. O Greep Effects of luteinizing hormone on progestin biosynthesis in the luteinized rat ovary *Endocrinology* 1964 *75* 488—500

Astwood F B The regulation of corpus luteum function by hypophysial luteotrophin *Endocrinology* 1941 *28* 309—320

Bassett D L The changes in the vascular pattern of the ovary of the albino rat during the estrous cycle *Amer J Anat* 1943 *73* 251—291

Burke G K. Kowalski and S Yu Effects of 3 5-cyclic nucleotides on membrane transport in isolated thyroid cells *Acta endocr* (Kbh.) 1971 *68* 183—195

Browning H C G A Larke and W D White Action of purified gonadotrophins on corpora lutea in the cyclic mouse *Proc Soc exp Biol* (N Y) 1962 *111* 686—690

Channing C P and C A Villee Luteinizing hormone Effects on uptake and metabolism of hexoses by luteinized rat ovaries *Biochim biophys Acta* (Amst) 1966 *115* 205—218

Christensen H N and J C Jones Amino acid transport models Renal resorption and resistance to metabolic attack *J biol Chem* 1962 *237* 1203—1206

Cole H H On the biological properties of mare gonadotropic hormone *Amer J Anat* 1936 *59* 299—331

Demers L M H R. Behrman and R. O Greep Effects of prostaglandins and gonadotrophins on luteal prostaglandin and steroid biosynthesis In *Advances in the Biosciences Vol 9* International Conference on Prostaglandins Ed Bergström S Pergamon Press/Vieweg Braunschweig 1973 701—707

Flint A. P F and R. M Denton Glucose metabolism in the superovulated rat ovary in vitro *Biochem J* 1969 *112* 243—254

Fuxe K. T Hökfelt C D Sundstedt K. Ahrén and L Hamberger, Amine turnover changes in the tubero-infudibular dopamine (DA) neurons in immature rats injected with PMS *Neuroendocrinology* 1972 *10* 282—300

Guillet G G and E G Rennels The experimental production of deciduomata in immature rats *Texas Rep Biol Med* 1964 *22* 78—93

Hashimoto I and W G Wiest Correlation of the secretion of ovarian steroids with function of a single generation of corpora lutea in the immature rat. *Endocrinology* 1969 *84* 873—885

Hausler C L. and P V Malven Interaction between prolactin LH neurohypophysial hormones and hysterectomy on luteal function in rats *Biol Reprod* 1971 *5* 262—269

examinations of the muscles studied (Botelho and Cander 1953 Desmedt and Hainaut 1967, 1968 Slomic *et al* 1968 Takamori 1971)

The present investigation was performed on human external intercostal muscles obtained during thoracic surgery Bundles of about 100 fibres were easily isolated with tendons more than 2 mm long at both ends. The contraction curve resulting when all the fibres in such a bundle were stimulated was recorded under isometric conditions before and after repetitive direct stimulation (10 Hz for 30 s) Membrane potentials were recorded with the intracellular technique. All preparations were examined histochemically after the experimental procedures

For comparison a series of similar experiments was performed on rat intercostal muscle

Methods

Experiments were carried out on external intercostal muscles obtained from

1) Patients undergoing thoracotomy for pulmonary tumours (4 patients) mitral stenosis (2 patients) or bronchiectasis (2 patients) and
2) Sprague Dawley rats of both sexes and weighing between 256 and 457 g The rats were anesthetized with an i.p injection of pentobarbitone (Nembutal, Abbott) 50 mg/kg b wt.

The muscles were taken in both cases between the 5th and 6th ribs near the anterior axillary line transferred to the experimental cell, and there dissected under a continuous flow of oxygenated Ringer's solution. The human muscles were kept in oxygenated Ringer's solution also during transport from the operation theatre The muscles could be kept under these conditions for up to 10 h without any apparent change in the physiological properties studied

Electrical recordings The experimental set up has been described in detail earlier (Hanson 1974) A new amplifier was used in the present investigation (grid current about 10^{-11} A linear over bandwidth 0—10 000 Hz voltage amplitude reduced to 70 % at 50 00 Hz) The fibres were stimulated by pulses of 0 1 to 0 2 ms duration delivered through a Ringer filled glass pipette

Mechanical recordings From the human muscles bundles of some 100 fibres could be dissected with sufficient tendons at both ends to permit fixation both to the floor of the experimental cell and to the isometric force transducer In the rat muscle preparations, the tendons being much shorter the tendon at one end of the muscle was left attached to the rib which was fixed to the floor of the experimental cell The tendon at the other end was carefully dissected and connected to the transducer The distances between the fixation points and the muscle fibres were always kept as short as possible. The preparations were kept horizontally and at an estimated *in situ* length.

The electronic device was the same as in the previous investigation (Hanson 1974)

Electrical stimuli were delivered through Ag AgCl electrodes placed along the fibres The stimulus strength was set at twice that which gave maximal twitch amplitude to a single shock of 1 ms duration

The standard programme for repetitive stimulation was 1 Hz for 10 s 10 Hz for 30 s 1 Hz for 10 s Contraction curves were recorded 1/2 2 3 1/2 and 5 min after the end of the 10 Hz sequence Tetanus stimulation was done in some preparations (50 Hz in human muscles 100 Hz in rat muscles)

Parameters measured

Intracellular recordings Resting membrane potential (E_R) height of the action potential (V) height of the negative after potential 3 or 2 ms after the beginning of the action potential in human and rat muscle fibres respectively (V_A) membrane potential at the same time (E_A) and the duration of the spike of the action potential (dur)

Contraction records Amplitude contraction time half relaxation time (*cf* Hanson 1974 Fig 1

Statistical methods The rank correlation test (*cf* Bennett and Franklin 1967 p 293) was used to determine whether there was any relation between the physiological properties and the fibre composition of the preparations The effect of repetitive stimulation was studied with variance analysis one way classification (*cf* Bennett and Franklin 1967 p 319) for contraction studies two-way classification *cf* Dixon and Massey 1957 p 163) for the parameters recorded intracellularly

TABLE I Results of intracellular recordings from human intercostal muscle fibres before and after repetitive stimulation (10 Hz for 30 s) All recordings from different fibres The values are the means (range) Preparations taken from 3 patients Temperature 37—38 C

	Before rep stim n = 106	Immediately after n = 23	5 min after n = 50
E_R mV	80 5 (71—93)	81 0 (74—94)	81 3 (72—90)
V_S mV	104 1 (91—119)	10? 5 (89—122)	106 0 (89—126)
Dur ms	1 50 (1 20—2 00)	1 67 (1 20—2 10)	1 47 (1 20—1 80)
V_A mV	6 7 (0—14)	7 7 (0—20)	7 8 (1—15)
E_A mV	73 8 (65—86)	73 2 (63—80)	73 6 (65—8?)

Solutions Ringer s solutions had the following composition (mM) Human muscle experiments NaCl 135 KCl 5 0 $CaCl_2$ 2 0 $NaHCO_3$ 15 Na HPO_4 1 0 MgCl 1 0 glucose 11 and tubocurarine chloride 20 μg/ml (Tubarine B W & Co)

Rat muscle experiments NaCl 140 KCl 5 0 $CaCl_2$ 2 0 $NaHCO_3$ 12 0 NaH_2PO_4 1 0 MgCl 1 0 glucose 11 and tubocurarine chloride 20 μg/ml

The solutions were continuously bubbled with 93 5 % O and 6 5 % CO_2 and led to the experimental chamber at a flow rate of 1000 ml/h via a heating coil In addition the solution in the bath was aerated with pure O The pH in the chamber ranged from 7 2 to 7 4 During recording the temperature was kept either at 22—24 C or at 37—38 C

Histochemical methods All preparations were frozen immediately after the experiments, cut in a cryostat and stained for succinic dehydrogenase (SDH Nachlas *et al* 1957 *cf* Pearse 1960) diphosphopyridine nucleotide diaphorase (DPN diaphorase Scarpelli *et al* 1958) and myofibrillar ATPase after incubation at pH 9 4 (Padykula and Herman 1955 *cf* Pearse 1960) Some preparations were also stained for glycogen (PAS Mowry and Millican 1952 modified by Nystrom 1968)

The fibres in the preparations were classified as either type I or type II according to their int nsity (low and high respectively) in the stain for myofibrillar ATPase (Dubowitz and Pearse 1960)

Results

A *Human muscles*

Intracellular recordings Intracellular recording from the same muscle fibres before and after repetitive stimulation (10 Hz for 30 s) revealed no discernible changes

Table I shows the results of recordings from 3 groups of previously unpenetrated muscle fibres two of which were stimulated repetitively (10 Hz for 30 s) Of the stimulated groups one was examined immediately (less than 15 s) after the end of the stimulation sequence and the other after 5 min rest

The mean duration of the action potential was 10 % greater in the just stimulated group than in the unstimulated group but the difference was not significant ($p >$ 0 05) Neither in the other parameters were there any significant differences between the groups Thus it was not possible to demonstrate any definite effect of repetitive stimulation on the intracellularly recorded potentials in human muscle fibres

The values for the respective parameters of the membrane potentials did not

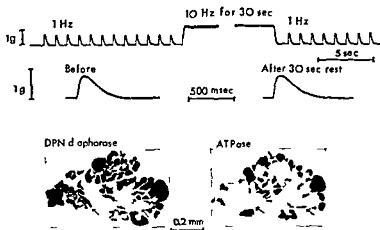


Fig 1 Contraction records of a human intercostal muscle preparation containing 92 % type I and 8 % type II fibres Temperature 37—38 C Contraction time 84 ms both before and after the sequence of repetitive stimulation This figure and Fig 2 4 and 5 include histochemical stains of the preparations used in the experiments Arrows indicate identical fibres

definitely diverge from the normal distribution

Contraction records The contractions of the fibre bundles taken at random from the external intercostal muscles were examined both at body and at room temperature The twitch was two to three times slower at room temperature while the developed twitch peak tension was approximately the same As was expected preparations which contained many type I fibres and few type II fibres (Fig 1) generally had longer contraction time than preparations with about the same amount of fibres of both types (Fig 2) In Fig 3 the contraction times of the

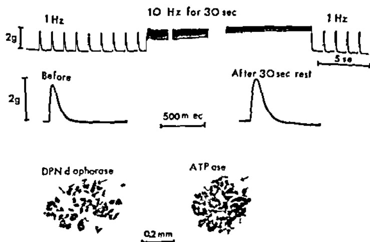


Fig 2 Contraction records of a human intercostal muscle preparation containing 4 % type I fibres and 53 type II fibres Temperature 37—38 C Contraction time 49 ms before and 60 ms after the sequence of repetitive stimulation.

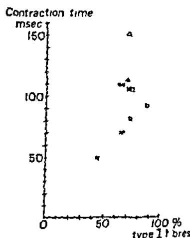


Fig 3 Contraction times plotted against fibre composition of 17 human external intercostal muscle preparations from 5 patients The different symbols denote different patients Temperature 37—38 C

human muscle preparations at body temperature are plotted against the percentage type I fibre content of the preparations The correlation coefficient was 0 79 ($p <$ 0 15) The half relaxation times also showed a tendency to be long in preparations with many type I fibres (correlation coefficient 0 81 ($p < 0$ 15)

Repetitive stimulation at 1 Hz for 10 s caused a slight decline of the twitch (mean 10 % n = 12) Stimulation at 5 Hz caused a partial fusion of the twitches which led to a slight increase in the peak tension of the first two or three twitches Thereafter, however the peak tension declined rapidly by on the average 30 %

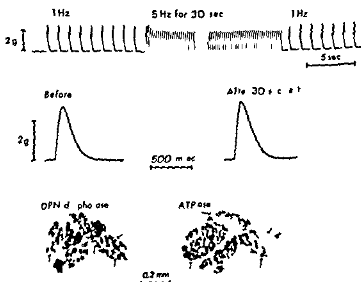


Fig 4 Contraction records of a human intercostal muscle preparation containing 67 % type I fibres and 33 % type II fibres Temperature 37—38 C Contraction time 77 ms before and 84 ms after the sequence of repetitive stimulation

TABLE II Twitch data of human intercostal muscle preparations before and after repetitive stimulation (10 Hz for 30 s) The values are the means (range) 12 preparations taken from 4 patients Temperature 37—38 C

	Before rep stim	30 s after
Contraction time ms	78 8 (42—109)	73 5 (46—109)
Half relaxation time ms	138 9 (63—272)	117 9 (71—176)

(n = 5 Fig 4) Thereafter only small changes were seen in the peak tension during the stimulation sequence The twitch increased to the original size when the stimulation frequency was reduced to 1 Hz

Stimulation at 10 Hz caused a marked fusion and initial increase in peak tension rapidly followed by a small but definite decrease (Fig 1 and 2) During the remainder of the 30 s stimulation sequence at 10 Hz no further changes were seen After reduction of the stimulation frequency to 1 Hz the amplitude of the twitch was about the same as at the beginning of the initial 1 Hz sequence (mean 88 % range 60—124 %) Neither was the time course of the twitch influenced (Table II)

After the stimulation series were completed the twitch tetanus ratio was determined in a few preparations and found to be on the average 0 24 (range 0 13—0 31 n = 7)

The effects of repetitive stimulation were essentially the same in all preparations both in those consisting predominantly of type I fibres (Fig 1) and in those with about the same amount of fibres of both types (Fig 2)

Thus the changes in the human muscle twitch produced by stimulation at 10 Hz were small in striking contrast to the marked staircase phenomenon seen in fast rat muscles in earlier (Hanson 1974) and present (Fig 5) investigations

B *Rat muscles*

Intracellular recordings Since the results in the human muscle experiments were to some extent unexpected also intercostal muscle fibres from the rat were examined to see whether the lack of changes during repetitive stimulation was specific for the species or was due to the fact that a muscle with such a special function was examined

Recording of membrane potentials from the same muscle fibres before and after repetitive stimulation (10 Hz for 30 s) showed the same type of changes as earlier observed in frog muscle fibres (Persson 1963 Hanson and Persson 1971) and in rat fast muscle fibres (Hanson 1974) The same procedure for statistical analysis of the effects of repetitive stimulation as in the case of human muscles was used for the rat muscles The results are presented in Table III It was found that the group of fibres penetrated immediately after stimulation at 10 Hz for 30 s had significantly higher amplitude of the negative after potential lower membrane potential at the same time (E_N) and longer duration of the spike of the action potential ($p < 0\,01$)

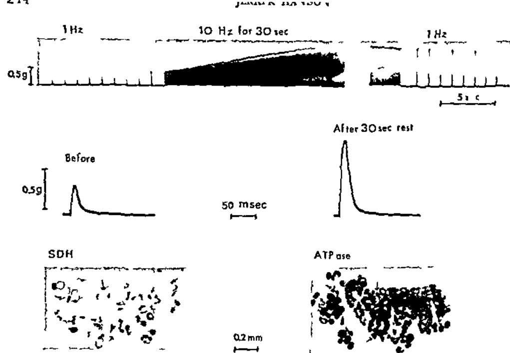


Fig 5 Contraction records of a rat intercostal preparation containing 87 % type II fibres and 13 % type I fibres Temperature 37—38 C Contraction time 10 ms before and 12 ms after the sequence of repetitive stimulation

as well as lower resting membrane potential and lower amplitude of the spike of the action potential ($p < 0.05$) than the previously unstimulated fibres The differences between the group of fibres examined after 5 min rest and the unstimulated group were small ($p > 0.05$), for the after potential ($p < 0.05$)

The mean resting membrane potential in previously unstimulated rat muscle fibres was 4 mV lower than in the corresponding group from man The duration

TABLE III Results of intracellular recordings from rat intercostal muscle fibres before and after repetitive stimulation (10 Hz for 30 s) All recordings from different fibres The values are the means (range) Preparations taken from 4 animals Temperature 37—38 C

	Before rep stim n = 210	Immediately after n = 111	5 min after n = 129
E_R mV	76 7 (70 – 87)	75 0 (70 – 86)	77 2 (70 – 84)
V_S mV	106 6 (85 – 123)	99 2 (86 – 119)	105 7 (87 – 120)
Dur ms	0 89 (0 65 – 1 10)	1 24 (0 75 – 1 80)	0 88 (0 65 – 1 40)
V_A mV	8 8 (4 – 17)	14 2 (6 – 24)	10 3 (5 – 18)
E_A, mV	67 9 (61 – 76)	61 1 (53 – 71)	6C 9 (58 – 4)

TABLE IV Twitch data of rat intercostal muscle preparations before and after repetitive stimulation (10 Hz for 30 s) The values are the means (range) 12 preparations from 4 animals Temperature 37—38 C.

	Before rep stim	30 s after
Contraction time ms	11.3 (10—13)	14 3 (10—17)
Half relaxation time ms	13 4 (10—20)	17 1 (11—23)

of the action potential was about half as long in the rat

Contraction records Experiments were performed at room temperature as well as at body temperature At room temperature the twitch was 2—3 times slower than at body temperature and the developed twitch peak tension about twice as high

At body temperature repetitive stimulation at 1 Hz for 10 s produced no changes in the twitch At 10 Hz however there was a strong increase in the twitch peak tension staircase phenomenon (Fig 5) which reached its maximum after 15—20 s stimulation (mean 274 % of the original amplitude range 205—366 %) During the remainder of the 30 s stimulation sequence at 10 Hz there was a slight decrease in the twitch amplitude (to on the average 239 % of the original value range 170—343 %) After reduction of the stimulation frequency to 1 Hz there were no further changes in the twitch amplitude Repetitive stimulation did not influence the time course of the twitch (Table IV Fig 5)

The twitch tetanus ratio which was determined at the end of the experiments in some of the preparations was on the average 0 07 (range 0 04—0 12 n = 9) at body temperature and 0 21 (range 0 13—0 34 n = 8) at room temperature

Histochemical investigations showed that the preparations consisted on the average of 84 % type II fibres (range 70—98 %) Furthermore the type I fibres were usually smaller than the type II fibres and therefore at least 75 % of the cross section area consisted of fibres strong in ATPase activity Thus the rat muscle preparations were more uniform than the human muscle preparations There was no demonstrable correlation between the variations in the fibre composition of the preparations and their physiological properties

Discussion

The measured values of the intracellularly recorded potentials in human intercostal muscle are on the whole in agreement with earlier findings in these muscles (Elmqvist *et al* 1960 1964 1968 Ludin 1969 Lipicky *et al* 1971) and also in other human muscles (Bolte *et al* 1963 Goodgold and Eberstein 1966 Brooks and Hongdalarom 1968 McComas *et al* 1968) The values measured in the rat intercostal muscle were about the same as those in other fast muscles of the rat (*cf* Hanson 1974) apart from the duration of the action potential which was somewhat longer in the preparations examined in this study

The resting membrane potential was on the average about 4 mV higher in human than in rat intercostal muscle fibres and the duration of the action potential about twice as long. No explanation for this difference is offered at present.

With regard to the after potential, the difference between human and rat intercostal muscles is not pronounced but in both cases the after potential is considerably smaller than in frog muscle fibres (Persson 1963, Hanson and Persson 1971). The cause of these differences is probably to be sought in the sizes of the transverse tubular systems and/or in the permeabilities of the tubular membrane (*cf.* Hanson 1974).

The contraction times of the human muscle preparations examined here are within the same range as those reported in earlier investigations of other human muscles (McComas and Thomas 1968, Buchthal and Schmalbruch 1970, Marsden and Meadows 1970) including recordings of contractions from single motor units (Sica and McComas 1971). The rat intercostal muscles were considerably faster than the human muscles. In cat intercostal muscles Andersen and Sears (1964) found fast and slow motor units with average contraction times of 25 and 47 ms respectively, most of the fibres thus being faster than the human muscle fibres examined here.

Previous animal experiments have demonstrated that the contraction speed is correlated to the myofibrillar ATPase activity (Barany 1967, Barnard *et al.* 1971, Burke *et al.* 1973, Kugelberg 1973). Some experimental findings indicate that this is also the case in human muscles (Buchthal and Schmalbruch 1970, Sica and McComas 1971). Data obtained in the present investigation support this theory. However, the twitch in the human muscle preparations is considerably slower than in rat soleus preparations (*cf.* Hanson 1974). This cannot be explained by differences in the fibre compositions since the rat soleus muscle consists of about 90 % type I fibres. Neither can the low contraction speed of the human muscles be explained by lower absolute values of myofibrillar ATPase activity, in any case not as far as can be judged from the histochemical stainings. It must be due to factors other than those investigated here.

As was expected, the twitch and the action potential were slower at room temperature than at body temperature both in rat and human muscles. In the fast rat muscle the amplitude of the twitch was also higher at room temperature than at body temperature (Hanson 1974, present investigation) which seems to be natural since the contractile elements are apparently activated over a longer period and since the elasticity of the series elastic component is probably lower at room temperature than at body temperature. In human muscles (present investigation) as well as in rat soleus muscle fibres (Hanson 1974), however, the amplitude of the twitch was about the same at room temperature as at body temperature despite longer twitch duration.

The behaviour of rat and human muscle preparations was markedly different during repetitive stimulation. The rat muscle preparations used in contraction studies consisted essentially of type II fibres and showed a marked increase of the

twitch amplitude (staircase phenomenon poststimulatory potentiation) without change in the time course of the twitch as was also found in an earlier study of the rat limb muscles (Hanson 1974) This phenomenon was not seen in human muscle preparations either in those which contained mainly type I fibres or in those which contained an equal number of type I and type II fibres Thus poststimulatory potentiation does not appear to occur in human intercostal muscle fibres A post stimulatory potentiation of the twitch of the adductor pollicis muscle in man after repetitive stimulation via the nerve to the muscle has however been described (Botelho and Cander 1953 Desmedt and Hainaut 1967 1968 Slomic *et al* 1968 Takamori *et al* 1971) The potentiation did not appear under ischemic conditions (Botelho and Cander 1953)

Close and Hoh (1968) pointed out that muscles which show a postimulatory potentiation of the twitch also show an increase of the twitch amplitude when the body temperature is lowered to room temperature and that muscles which do not show poststimulatory potentiation of the twitch do not either show temperature dependent variations in the twitch amplitude The same observations have been made in the present study of intercostal muscles from man and rat, as well as in an earlier study of rat limb muscles (Hanson 1974) Muscles which show post stimulatory potentiation also have lower twitch tetanus ratio than those in which no potentiation occurs It appears probable that these simultaneously occurring properties are dependent on the same factors

The intracellularly recorded potentials in human muscles were not markedly changed during repetitive stimulation while in the rat intercostal muscles the same type of change was seen as earlier observed in fast limb muscle of rat (Hanson 1974) and in frog muscle fibres (Hanson and Persson 1971) Thus the human muscle fibres resemble in this respect as well the slow soleus muscle of the rat (Hanson 1974)

This investigation was supported by a grant from Karolinska Institutet My thanks are due to Miss Birgitta Hedberg and Miss Birgitta Lindegren Department of Neurology Karolinska sjukhuset, who performed all the histochemical work in this investigation Professor Eric Kugelberg kindly placed the resources at my disposal

References

ANDERSEN P and T A SEARS The mechanical properties and innervation of fast and slow motor units in the intercostal muscles of the cat *J Physiol* (Lond) 1964 *173* 114—129

BARANY M ATPase activity of myosin correlated with speed of muscle shortening *J gen Physiol* 1967 Suppl *50* part 2 197—218

BARNARD R J V R EDGERTON T FURUKAWA and J B PETER Histochemical biochemical and contractile properties of red white and intermediate fibers *Amer J Physiol* 1971 *220* 410—414

BENNETT C A and N L FRANKLIN *Statistical analysis in chemistry and the chemical industry* John Wiley & Sons Inc New York 1967

BOLTE H D G RIECKER and D ROHL Messungen des Membranpotentials an einzelnen quergestreiften Muskelzellen des Menschen in situ *Klin Wschr* 1963 *41* 356—359

BOTELHO S Y and L CANDER Post tetanic potentiation before and during ischemia in intact human skeletal muscle *J appl Physiol* 1953 *6* 221—228

BROOKS J E and T HONGDALAROM Intracellular electromyography Resting and action potentials in normal human muscle *Arch Neurol* (Chic) 1968 *18* 291—300

BUCHTHAL F and H SCHMALBRUCH Contraction times and fibre types in intact human muscle *Acta physiol scand* 1970 79 435—452
BURKE R. E D N LEVINE P TSAIRIS and F E. ZAJAC Physiological types and histochemical profiles in motor units of the cat gastrocnemius *J Physiol* (Lond) 1973 234 723—748
CLOSE R. I and J F Y HOH Influence of temperature on isometric contractions of rat skeletal muscles *Nature* (Lond) 1968 217 1179—1180
DESMEDT J E. and K. HAINAUT Modifications des proprietes contractiles du muscle strie au cours de la stimulation electrique repetee de son nerf moteur chez l'Homme normal *C R Acad Sci* (Paris) 1967 264 363—366
DESMEDT J E and K HAINAUT Kinetics of myofilament activation in potentiated contraction staircase phenomenon in human skeletal muscle *Nature* (Lond) 1968 217 529—532
DIXON W J and E J MASSEY *Introduction to statistical analysis* 2nd ed McGraw Hill Book Co New York 1957
DUBOWITZ V and A G E. PEARSE A comparative histochemical study of oxidative enzyme and phosphorylase activity in skeletal muscle *Histochemie* 1960 2 105—117
ELMQVIST D and E. H LAMBERT Detailed analysis of neuromuscular transmission in a patient with the myasthenic syndrome sometimes associated with bronchogenic carcinoma. *Mayo Clin Proc* 1968 43 689—713
ELMQVIST D W W HOFMANN J KUGELBERG and D M. J QUASTEL, An electrophysiological investigation of neuromuscular transmission in myasthenia gravis *J Physiol* (Lond.) 1964 174 417—434
ELMQVIST D T R. JOHNS and S THESLEFF A study of some electrophysiological properties of human intercostal muscle. *J Physiol (Lond.) 1960 154 602—607*
GOODGOLD J and A. EBERSTEIN Transmembrane potentials of human muscle cells in vivo *Exp Neurol* 1966 15 338—346
HANSON J The effects of repetitive stimulation on the action potential and the twitch of rat muscle *Acta physiol scand* 1974 90 387—400
HANSON J and A. PERSSON Changes in the action potential and contraction of isolated frog muscle after repetitive stimulation *Acta physiol scand* 1971 81 340—348
KUGELBERG E Histochemical composition contraction speed and fatiguability of rat soleus motor units *J neurol Sci* 1973 20 177—198
LIPICKY R. J S H. BRYANT and J H. SALOMON Cable parameters sodium, potassium chloride and water content, and potassium efflux in isolated external intercostal muscle of normal volunteers and patients with myotonia congenita. *J clin Invest* 1971 50 2091—2103
LUDIN H. P Microelectrode study of normal human skeletal muscle *Europ Neurol* 1969 2 340—347
MARSDEN C D and J C MEADOWS The effect of adrenaline on the contraction of human muscle *J Physiol* (Lond 1970 207 429—448
McCOMAS A J and H C THOMAS Fast and slow twitch muscles in man *J Neurol Sci* 1968 7 301—307
McCOMAS A. J K MROZEK D GARDNER MEDWIN and W H. STANTON Electrical properties of muscle fibre membranes in man. *J Neurol Neurosurg Psychiat* 1968 31 434—440
MOWRY R. W and R. C MILLICAN A histochemical study of the distribution and fate of dextran in tissues of the mouse. *Amer J Pathol* 1952 28 522
NACHLAS M M K C TSOU E DE SOUZA C S CHENG and A M SELIGMAN Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole *J Histoch m Cytochem* 1957 5 420—436
NYSTRÖM B Histochemistry of developing cat muscles *Acta neurol scand* 1968 44 405—439
PADYKULA H A. and E HERMAN The specificity of the histochemical method for adenosine triphosphatase *J Histochem Cytochem* 1955 3 170—183
PEARSE A. G E. *Histochemistry theoretical and applied* J & A C [illegible] ill Ltd. London 1960
PERSSON A. The negative after potential of frog skeletal [illegible] scand 1963 58 Suppl. 205 [illegible]
SCARPELLI D G R. HESS and A. G E PEARSE The cyt[illegible] enzymes *J biophys biochem Cytol* 1958 4 747—752
SICA R. E. P and A J McCOMAS Fast and slow twitch [illegible] *Neurosurg Psychiat* 1971 34 113—120
SLOMIC, A. A. ROSENFALCK and F BUCHTHAL, [illegible] and myasthenic muscle *Brain Res* 1968 10 [illegible]
TAKAMORI M., L. GUTMANN and S R. SHANE [illegible] *Arch Neurol* (Chic.) 1971 25 535—546

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From the Department of Clinical Neurophysiology and Department of Neurology
University Hospital Gentofte Denmark

Decremental Conduction in Normal Human Nerves Subjected to Ischemia?

By

V Kamp Nielsen and T Kardel

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Abstract

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The effect of ischemia on the maximal orthodromic sensory and motor nerve conduction was studied in the digit 1 elbow segment of the median nerve in 24 normal subjects Action potentials were recorded at 2 3 or 4 sites along the nerve The latency of the potentials increased as a function of the duration of ischemia and of the distance from the point of stimulation The earliest indication of an impaired nerve function was a decrease in the sensory action potential amplitude During ischemia the length of the nerve segment, through which the potential was propagated gradually became shortened This was seen in sensory as well as in motor nerves The sensory threshold to electrical stimuli (digit 3) had a two phasic course During 15 min of ischemia it was almost stationary despite a severe reduction of the conduction velocity and of the potential amplitude Then a rapid increase took place coinciding with the extinction of the potential recorded at the elbow just below the occluding cuff The conduction parameters were normalized shortly after reestablishment of the circulation in the arm The results indicate that action potentials become conducted with decrement during ischemia

In 1959 Lorente de No and Condouris reinstated the hypothesis of non linear or *decremental nerve conduction This had originally been formulated by Szpilman* and Luchsinger in 1881 and was widely accepted for several decades when it became opposed by several authors and finally disappeared from the literature Lorente de No and Condouris reevaluated the arguments for and against the hypothesis and added further experimental evidence in favour of decremental nerve conduction They concluded that peripheral nerves can be expected to conduct with decrement when the axon membrane potential becomes reduced or the nerve is subjected to the action of local anesthetics This statement has only been commented upon in a few physiological reviews (Davis 1961 Frank and Fuortes 1961)

Impairment of the axon membrane function may result from a metabolic intoxication as for example in progressive chronic renal failure where decremental nerve conduction has been suspected (Nielsen 1973 b 1974) However in uremia

the pattern of nerve conduction is often obscured by an ascending demyelination which predominantly affects the most distal part of peripheral nerves as opposed to a generalized affection of the axon membrane by uremic toxin(s) We therefore decided to study the nerve conduction during a selective impairment of the axon membrane function In normal subjects a long segment of the median nerve was subjected to short lasting ischemia This was considered as a proper model since it has been shown that ischemia produces a gradual depolarisation of the membrane (Lorente de No 1947 Maruhashi and Wright 1967) without any significant morphological alterations of the nerve distal to the compression (Ochoa *et al* 1971) Our results provide evidence that the impulse propagation becomes non linear under these circumstances

A preliminary report of this investigation was presented at the 20 congress of Scandinavian Neurologists June 1972 Oslo Norway (Nielsen and Kardel 1972)

Material and Methods

Twenty four normal persons volunteered in the investigation the purpose and procedure of which was explained to them in advance There were 12 female and 12 males, in average 36 years of age (range 17—71 years)

Electrophysiological examination The *sensory* conduction in the *median nerve* was measured as described by Buchthal and Rosenfalck (1966) Sensory fibres in *digit I* were stimulated through ring electrodes placed on the distal phalanx The stimulus strength was about 15 × the pre ischemic sensory threshold stimulus strength and was constant throughout the experiment Evoked sensory action potentials were recorded at 2—4 sites along the nerve between the wrist and the elbow by needle electrodes placed close to the nerve the motor threshold stimulus strength being less than 1 0 mA The remote electrode was placed at a transversal distance of 30—40 mm from the near nerve electrode The action potentials were displayed on a 3-channel electromyograph (DISA) The arm was heated throughout the examination by a heating element and the skin temperature at the wrist and the elbow measured regularly with a thermocouple was never below 34 C and deviated less than ±1 C during the examination

The following variables were analyzed 1) The *latency* (ms) from the stimulus artefact to the first positive peak of the evoked sensory action potential 2) The *conduction velocity* (m in the distal (digit I wrist) and the proximal (wrist-elbow) segment or in 2 or 3 subsegments between wrist and elbow 3) The *temporal dispersion* (ms defined as the difference between the latencies to the first and second positive peak of the triphasic action potential recorded at the wrist 4) The peak to-peak potential *amplitude* (μV 5) In addition the *sensory threshold* to electrical stimuli (mA) transmitted through a ring electrode was recorded distally on *digit 3* as the weakest impulse that could be discerned by random stimulation

The *motor* conduction was measured after stimulation of the nerve through the electrodes placed at the wrist, the forearm and the elbow with recording of the evoked muscle action potential by concentric needle electrodes placed in the end plate zone of the abd poll brev muscle Each series lasted 1—2 min

Procedure Several recordings were made before induction of ischemia as *reference* values ("pre-ischemic value A pneumatic cuff 12 cm wide) around the upper arm about 5 cm proximal to the elbow electrodes, was rapidly inflated to a pressure well above the systolic blood pressure and never below 180 mm Hg Nerve conduction measurements were made every 2—3 min during ischemia. The sensory threshold (digit 3) was recorded at least every 5 min After 30 min the cuff was *deflated and recordings were made* as soon as possible and at regular intervals for 20—30 min post ischemic values)

In six patients the wrist-elbow segment was subdivided into 2 or 3 subsegments, inserting needle electrodes close to the nerve at about equidistant intervals. Hence it was possible to record the same action potential along the nerve at wrist, forearm and elbow When a fourth recording electrode was used each series of measurements were performed within one minute (wrist forearm I forearm II-elbow

The ratio between the peak to-peak amplitude and the noise level of the base line gradually

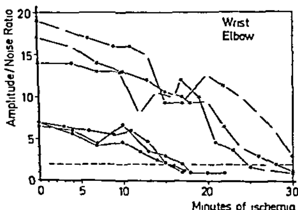


Fig 1 Changes in the ratio between the peak-to-peak amplitude of action potentials and the noise level of the base line during 30 min of ischemia. The action potentials were recorded at the wrist and at the elbow following supramaximal stimulation of sensory nerve fibers in digit 1 in three normal subjects Each ratio was calculated from measurements on photographic recordings of 20 superimposed action potentials ——— = Critical ratio

became reduced towards 1 during the experiment (Fig 1) but the ratio was > 2 for more than 13 min at the elbow and for more than 22 min at the wrist. The average amplitude of the last recorded sensory action potential during ischemia was 2.2 μV (range 0.8—6.0 μV) at the wrist and 2.3 μV (range 1.0—4.8 μV) at the elbow

Analysis of data Changes in the nerve conduction parameters during and after ischemia were expressed as relative values compared with the pre ischemic observations (Table I and II) For each 3 min period the average relative change of all observations recorded within the period was calculated The significance of differences between the relative change of two variables within a period was tested with the Student's t test. The course of the changes in the parameters during and after ischemia described a curve with a more or less steep slope Hence the scatter of observations within a 3 min interval showed a systematic time dependent variation This strengthens the statistical test of significance as it tends to reduce the difference between two variables but on the other hand reinforces the significance of a difference when present.

Results

Sensory conduction velocity

During ischemia the sensory conduction velocity was reduced at a greater rate in the wrist elbow segment than in the digit wrist segment (Fig 2) The average reduction in the two segments deviated significantly after 6—9 min $p < 0.001$ (Table I) Thus 80 % of the pre ischemic value was reached about 12 min (10—15 min) and about 21 min (18—24 min) after onset of ischemia in the proximal and distal segments respectively The lowest conduction velocity during ischemia averaged 65—70 % of the pre ischemic value *After ischemia* the recovery of the conduction velocity was more delayed in the proximal segment Thus 90 % of the pre ischemic conduction velocity was regained after about 5 min (2—10 min) and after about 18 min (8—30) in in the distal and proximal segments respectively and the last recorded value 20—30 min after ischemia averaged 99 % (92—107 %) and 92 % (88—98 %) respectively

Subdivision of the digit l-elbow nerve segment into three or four sub egments gave consistent results in all experiments A typical example is shown in Fig 3 The

TABLE I The relative change in the sensory conduction velocity the potential amplitude and the

	Conduction velocity	
	Dig 1 wrist	Wrist-elbow
Pre ischemic mean value±S E (n)	42 1±0 66 (24) m/s	63 5±0 81 (22) m/s
During ischemia		
	% ±S E (n)	% ±S E (n) t
0– 3 min	100±0 2 (28)	99±0 3 (24) 1 55
3– 6	98±0 5 (28)	97±0 6 (26) 0 75
6– 9	96±0 5 (22)	91±0 8 (19) 6 42***
9–12	94±0 5 (30)	82±1 2 (26) 14 81***
12–15	92±0 6 (27)	74±1 0 (20) 15 22***
15–18	87±0 8 (30)	62±2 8 (6) 8 42***
18–21	82±1 2 (22)	
21–24	76±1 3 (28)	
24–27	71±1 6 (16)	
27–30	72±2 4 (9)	
After ischemia		
0– 3 min	85±1 4 (25)	76±1 1 (15) 5 10***
3– 6	90±1 0 (28)	81±0 8 (21) 6 93***
6– 9	93±1 1 (26)	84±0 7 (23) 6 98***
9–12	94±1 0 (26)	87±0 7 (19) 5 18**
12–15	94±1 0 (24)	88±0 6 (19) 5 74***
15–18	97±1 4 (18)	90±0 9 (14) 4 71***
18–21	96±1 4 (18)	92±0 6 (16) 3 27**
21–24	97±1 2 (21)	91±0 7 (18) 4 30***
24–27	98±1 3 (10)	92±0 7 (7) 3 99**
27–30	98±1 2 (18)	93±0 7 (14) 3 64***

The table comprises all measured values In some persons two recordings were made within one period In 2 persons no recordings were made at the elbow and in another 22 persons it was not always possible to record a suitable action potential at the elbow After 15 min of ischemia it became impossible in most of the persons Action potentials which could not be discriminated from the base line noise level were excluded —The sensory threshold (digit 3) was measured in a total of 16 persons t Student's t test of differences between distal and proximal segments * = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

rate of slowing of the conduction velocity was determined (1) by *the duration* of ischemia and (2) by *the distance* of the subsegment from the point of stimulation (Fig 4) *i e* the steepest curve was always recorded in the forearm (*forearm II*) elbow segment most remote from digit 1 After about 15 min of ischemia the action potentials gradually disappeared in a distal ward direction from the elbow to the wrist Fig 5 shows *the latency* (Lat) of the potentials at wrist forearm I forearm II, and elbow as a function of the pre ischemic latencies (Lat_0) and with the duration of ischemia as a parameter The curved deviation from the straight line Y = X (*time* = 0) illustrates the increase in latency as a function of the duration of ischemia and of the length of the nerve segment through which the action potential is propagated A mathematical analysis of the curve is presented in the appendix

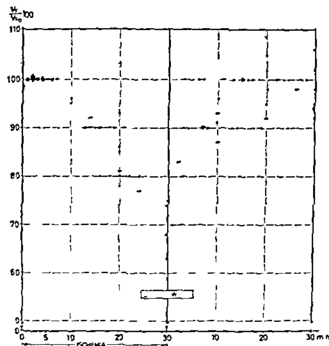
sensory threshold during and after ischemia in normal subjects

Amplitude		Sensory threshold
Wrist	Elbow	Digit 3
28 6±2 28 (24) μV	7 9±0 45 (22) μV	3.5±0 21 (16) mA
/ ±S E (n)	±S E (n) t	Ts/Ts ± S E (n)
95±1 4 (28)	90±1 7 (24) 2 10*	1 24±0 10 (10)
88±1 7 (28)	84±2 4 (26) 1 17	1 29±0 14 (10)
83±2 4 (22)	75±3 4 (19) 2 06*	1 19±0 08 (13)
75±2 1 (30)	48±3 4 (26) 6 74**	1 15±0 10 (14)
63±2 7 (27)	30±2 4 (20) 8 89*	2 15±0 48 (10)
47±3 2 (30)	18±1 9 (6) 7 65***	3 98±1 01 (15)
31±3 0 (22)		7 65±1 35 (15)
18±2 4 (28)		9 06±1.26 (13)
11±2 5 (16)		12 14±1 58 (16)
9±2 3 (9)		11 37±1 17 (11)
49±2 8 (25)	60±4 3 (15) −2 05	1 55 (2)
56±3 5 (28)	67±3 6 (21) −2 30	1 55±0 09 (10)
69±3 0 (26)	78±3 1 (23) −2 13	1 29±0 06 (13)
69±3 5 (26)	77±3 7 (19) −1 57	1 28±0 06 (10)
77±3 5 (24)	78±3 1 (19) −0 31	1 16±0 01 (7)
78±4 9 (18)	78±3 6 (14) 0 08	1 17 (4)
85±4 4 (18)	83±3 6 (16) 0 22	1 08±0 03 (6)
83±3 9 (21)	86±3 6 (18) −0 48	1 11 (1)
90±5 0 (10)	89±5 5 (7) 0 12	0 94 (4)
95±3 7 (18)	97±4 5 (14) −0 24	1 13±0 15 (7)

TABLE II The average changes in the latency to the 1st and 2nd positive peak of the sensory action potential recorded at the wrist during ischemia in normal subjects

	1 st peak	2nd peak	
Pre ischemic mean value±S E. (n)	2 9±0 06 (24) ms	4 2±0 09 (23) ms	
Duration of ischemia	Mean±S E (n)	Mean±S E (n)	t
0− 3 min	0 00±0 01 (28)	0 02±0 01 (26)	−1 55
3− 6	0 06±0 01 (28)	0.20±0 07 (28)	−1 92
6− 9	0 09±0 01 (2?)	0 20±0 03 (20)	−3 04
9−12	0 17±0 0? (30)	0 35±0 04 (29)	−4 44 **
12−15	0 26±0 02 (27)	0 55±0 05 (25)	−5 10 *
15−18	0 43±0 03 (30)	0 88±0 05 (28)	−7 35 *
18−21	0 62±0 05 (22)	1 39±0 11 (16)	−6 14*
21−24	0 9?±0 06 (28)	1 78±0 12 (15)	−6 53 *
24−27	1 20±0 09 (16)	1 83 (3)	
27−30	1 19±0 16 (9)		

t = Student's t test of differences between 1st and 2nd peak, ** = $p < 0.01$ = $p < 0.001$



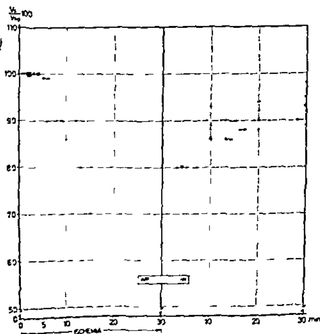


Fig. 2. Sensory conduction velocities in distal (Fig. 2 A, 24 persons) and proximal (Fig. 2 B, 22 persons) segments of the median nerve recorded during and after 30 min of ischemia in normal subjects 17—71 years of age. Each conduction velocity (Vs) was expressed as the percentage value of the pre-ischemic conduction velocity (Vs0). The difference between the two plots is analyzed in Table I.

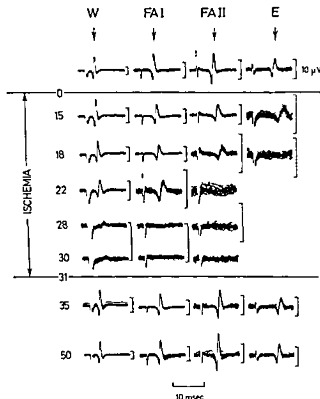


Fig 3 Sensory action potentials recorded along the median nerve following supramaximal stimulation of nerve fibers distally in digit 1 during and after ischemia. W = wrist FA I = forearm I FA II = forearm II E = elbow Each potential represents 20 superimposed action potentials The figures in the left column indicate minutes At 0 a pneumatic cuff placed around the upper arm was inflated to 200 mm Hg The cuff was deflated at 31 min The temperature at the wrist forearm and the elbow was about 35 C and decreased 0 5 C during the ischemic period EMG 345 female 20 yrs

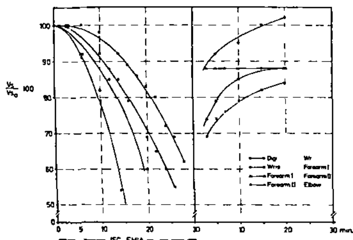
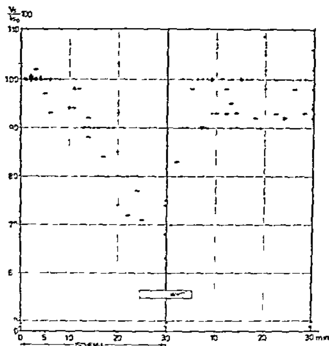


Fig 4 Relative change in the sensory conduction velocity in four subsegments of the median nerve during and after ischemia. EMG 345 female 20 yrs



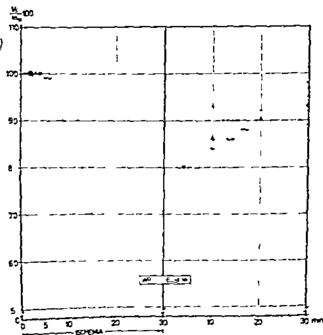


Fig. 2. Sensory conduction velocities in distal (Fig. 2 A 24 persons) and proximal (Fig. 2 B 22 persons) segments of the median nerve recorded during and after 30 min of ischemia in normal subjects, 17—71 years of age. Each conduction velocity (V_t) was expressed as the percentage value of the pre-ischemic conduction velocity (V_{t0}). The difference between the two plots is analyzed in Table I.

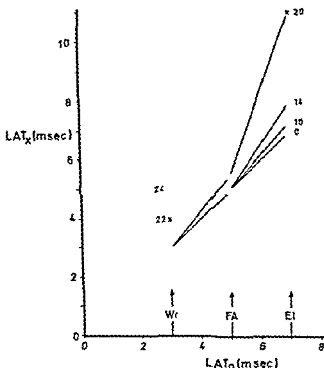


Fig 7 The latency (Lat_x) of the evoked muscle action potentials (abd poll brevis) following stimulation of the median nerve at wrist (W) forearm (FA) and elbow (E) at selected intervals after onset of ischemia expressed as a function of the pre ischemic latencies (Lat_0) and with the duration of ischemia as a parameter Figures above the curves indicate minutes after onset of ischemia After 20 min it was not possible to evoke an action potential by stimulation at the forearm and elbow while the latency following stimulation at the wrist had not yet increased EMG 287 male 26 yrs

The sensory action potential

The *temporal dispersion* of action potentials recorded at the wrist gradually increased during the ischemic period This was seen from the divergent increases of the latencies to the 1st and 2nd positive peaks of the potential which differed significantly after 6—9 min of ischemia $p < 0.01$ (Table II) After 18 min it became increasingly difficult to discern the 2nd positive peak The temporal dispersion rapidly normalized after deflation of the cuff

The potential *amplitude* decreased from the beginning of the ischemia After about 10 min of ischemia a rapid reduction was recorded more pronounced at the

TABLE III Th calculated decrement factor μ of the equation $L_T = L_0 e^{\mu L_0 T^2}$ (see appendix) and the mean d ff rence between observed and calculated latencies (L = latency (10 ms) T = minutes of ischem a)

EMG no	Number of potent als	μ	(S D)	Mean difference	S D)
345	38	0 26 10⁻⁴	(0 16 × 10)	0 04 ms	(0 15)
34?	37	0 ?4 10	(0 34 × 10⁻)	0 0? m	(0 20)
347	38	0 18 10⁻⁴	(0 27 × 10)	−0 01 ms	(0 18)

elbow than at the wrist (Table I) An action potential could be recognized for about 15 min (10—18 min) at the elbow and at the wrist for about 26 min (16—30 min) In four subjects a potential was still present at the wrist when the cuff was deflated after 30 min Then within a few minutes the amplitude reached 50 % of the pre ischemic value which was regained 20—30 min after ischemia There was no difference in the rate of recovery of the potential amplitude at the wrist and the elbow at the 1 % significance level

The sensory threshold

The course of the sensory threshold (digit 3) was *two phasic* During the first 15 min of ischemia the threshold was almost stationary in spite of a severe reduction of the potential amplitude at the elbow and of the conduction velocity in the wrist elbow segment (Table I) After 15 min a steep rise in the threshold was recorded. In all 16 persons examined this coincided with the time when the action potential disappeared at the elbow A few minutes after deflation of the cuff the threshold was practically normal. Fig 6 illustrates a typical course

The motor conduction velocity

Identical results were obtained in three experiments The motor latency increased earlier and more rapidly when motor nerve fibers were stimulated at the elbow than at the forearm or the wrist *i e* at a shorter distance from the muscle (Fig 7) After about 20 min the muscle could no longer be activated by stimulation at the forearm or the elbow while an action potential of near normal amplitude and only a lightly prolonged latency was still recorded when stimulating at the wrist. The block of the motor nerve conduction was preceded by a sudden decrease in the amplitude of the evoked muscle action potential

Discussion

The earliest report relating to the nerve function during ischemia was given by Nicolaus Steno (1667) who produced a reversible paralysis of the hind limbs in dogs by temporary ligation of the descending aorta (the Steno experiment) The modern literature has been reviewed by a.o Richards (1951) and Lundborg (1970)

The present study indicates that the nerve conduction velocity decreases as a function of the distance from the point of stimulation when a normal nerve is subjected to ischemia. In motor fibers the increase in the latency to the abd. poll. brevis muscle became comparatively greater the longer the conduction distance Eventually action potentials from the elbow died out along the nerve at a time when a near normal muscle response was evoked by impulses from the wrist (Fox and Kenmore 1967 Ruskin *et al* 1967 In sensory fibers the latencies to action potentials recorded at different levels along the nerve fitted in with curves computed from a relatively simple empirical equation which takes into account the preischemic

conduction time (*the conduction distance*) *and the duration of ischemia*, reflecting the degree of impairment of the axon membrane function (Lorente de No (1947) Wright (1947) Shanes (1958)) The deviations between measured and computed latencies (Table III) were small enough to suggest that a relatively *uniform temporo spatial slowing of the rate of the impulse propagation takes place during ischemia* The sensory potential *amplitude* decreased immediately after the blood flow was arrested while the *temporal dispersion* did not increase significantly until after 6—9 min of ischemia The *sensory threshold* (digit 3) was almost unchanged for about 15 min (Lewis Pickering and Rothchild (1931)) and a marked rise was not recorded until the elbow potential had almost disappeared

These findings are consistent with the hypothesis of decremental impulse propagation during impaired nerve membrane function (*Lorente de No and Condouris* (1959)) To our knowledge this hypothesis has not been tested specifically through in vivo studies in human nerves but the possibility was briefly mentioned by Castaigne *et al* (1972) However decremental impulse propagation is principally a *single fiber* phenomenon wheras this study concerns changes in the latency and amplitude of *compound* action potentials from a whole nerve For this reason certain sources of error and alternative explanations of our observations must be considered

In keeping with our results previous studies have shown that the shorter the conduction distance the greater is the resistance to ischemia (Magladery *et al* (1950) Cathala and Scherrer (1963) Fox and Kenmore (1967) Caruso *et al* (1973)) *Since the shortest conduction distance is usually* recorded in a distal nerve segment this is often considered more resistant to ischemia than proximal nerve segments This might be due to differences in the amount of available oxygen or in the diffusion rate of oxygen *e g* due to differences in the nerve fiber diameter Differences in temperature proximally and distally might also influence the measurements This interpretation however is inconsistent with the observation that distal muscles become inexitable far earlier than proximal muscles when the nerve is stimulated proximally but distal to the compression (Bentley and Schlapp 1943 Kugelberg 1944) In our study a subdivision of the wrist-elbow segment provided evidence that the greater resistance to ischemia was *not* a particular feature characteristic of the digit wrist segment (Fig 4) Moreover, the observations were principally analogous whether stimulating afferent fibers in digit 1 or efferent fibers at the elbow when the conduction distance is taken into account The temperature gradient over the skin was practically constant during the experiment and the heat loss was not greater in the proximal than in the distal part of the arm.

Due to the growing temporal dispersion of the compound sensory action potential the 1st positive peak may not represent the same fibers when recorded at different levels along the nerve or when recorded at the same level by increasing duration of ischemia Thus theoretically the 1st peak might come to represent *slower* conducting fibers at the elbow than at the wrist if ischemia resulted in a conduction block primarily affecting the fastest conducting fibers Assuming that the slowing

of the conduction velocity during ischemia were linear the presumed error in latency measurements to elbow potentials would be about 1 3 ms after 15 min of ischemia (ref Fig 5) Thus the 1st peak of the wrist potential would correspond to a point on the base line 1 3 ms *in front* of the observed peak of the elbow potential suggesting a block of all the fastest fibers However this is incompatible with the evidence presented by Lorente de No and Condouris (1959) showing that slower conducting fibers are affected earlier than fast fibers during experimental impairment of the axon membrane function which is in agreement with other reported observations during ischemia (see Lundborg 1970) In our study this was also indicated by the relatively greater increase of the latency to the 2nd than to the 1st peak of the potential (Table II) Furthermore, under normal conditions the 1st peak of compound sensory action potentials come to represent slightly faster conducting fibers when recorded at more proximal levels (Buchthal and Rosenfalck 1966) Therefore the observed increase in latency may in fact have been underestimated and more so the longer the conduction distance and the duration of ischemia. Hence the growing relative increase of the latency to potentials at successive recording sites (wrist forearm I forearm II-elbow) favored a *gradual* decrease of the conduction velocity along the nerve rather than a block of fibers This was supported by the parallel slowing but in the opposite direction when motor fibers were stimulated at different levels

It appears improbable that morphological changes of the examined nerve segments influenced the measurements since the nerve conduction was restored within minutes after ischemia. Moreover Ochoa *et al* (1972) showed that ischemic nerve lesions were restricted to the region under the edges of the cuff

As to technical sources of error the study demonstrated that the ratio between the noise level of the base line and the potential amplitude at the elbow was not critical until after 13—15 min of ischemia while the difference between changes in the distal and proximal conduction velocity became statistically significant already after 6—9 min When possible action potentials were recorded with a time base of 0 25 ms/mm This enabled a discrimination of changes of 0 1 ms and often of 0 05 ms As shown in Fig 5 the latency to the wrist potential should be about 0.3 ms longer than observed already after 10 min of ischemia if the slowing of the nerve conduction were linear This is at least 3 times the limit of discrimination

From these considerations we assume that our latency measurements are valid Hence the most likely explanation of our results is that nerve fibers when subjected to ischemia exhibit the phenomenon of decremental impulse propagation prior to a block of conduction

The potential amplitude is determined by the position of the electrode and the number of active fibers (Buchthal and Rosenfalck 1966) the temporal dispersion of the potential (Nielsen 1973 a) and the resting membrane potential of the single fibers Our observations were hardly influenced by changes in the electrode position as the potential amplitude was restored to normal after deflation of the cuff The temporal dispersion did not increase until after 6—9 min of ischemia and a re

duction in the number of active fibers is probably a late phenomenon. Therefore by exclusion the initial decrease in amplitude suggests a reduction of the single fiber membrane potential. This is possibly a precondition to decremental nerve conduction which was not demonstrable until 6—9 min after onset of ischemia.

During the first 15 min of ischemia the sensory threshold to electrical stimuli was widely unaffected by the severe reduction of the potential amplitude at the elbow and of the conduction velocity in the wrist elbow segment, which is normally the same in fibers from digit 1 and 3 (Nielsen 1973 a). This suggests that the threshold stimuli are conducted to the CNS in a normal manner as long as the threshold potential is able to depolarize the unaffected nerve fibers proximal to the occluding cuff. The electrophysiological events at the proximal border of the cuff may therefore exemplify *incremental* conduction as described by Lorente de Nó and Condouris (1959).

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Appendix

Assuming that ischemia leads to a uniform temporo-spatial sequence of changes of the nerve conduction an empirical mathematical formula, covering all measured latencies (L) at four recording sites during 30 min of ischemia (T) was set up. Based on the curves a likely expression would be

$$L_T = L_0^{F(T)} \qquad (1)$$

in which L_0 denotes the latency (ms) to a given recording site before ischemia. From an analysis of the exponent it was concluded that the increase of the power of T above the second degree infered no improvement and that the first degree coefficient was comparatively small so that the exponent could be reduced to $1 + kT^2$. However, k gradually increased with increasing L_0. This was avoided by introducing L_0 in the exponent

$$L_T = L_0^{1 + \mu L_0 T^2} \qquad (2)$$

where μ, a constant, was independent of L_0.

A μ value was calculated for each experiment excluding observations during the first 9 min of ischemia. To test the quality of the fit the mean deviation and the standard deviation between observations and computed values was calculated. Equation (2) gave standard deviations between 0.15 and 0.20 ms (Table III). (A complete set of data is available on request.)

References

Bentley, F. H. and W. Schlapp: Experiments on the blood supply of nerves. *J. Physiol.* (Lond.) 1943. *102*. 62—71.

Buchthal, F. and A. Rosenfalck: Evoked action potentials and conduction velocity in human sensory nerves. *Brain Res.* 1966. *3*. 1—122.

Caruso, G., O. Labianca and E. Ferrannini: Effect of ischaemia on sensory potentials of normal subjects of different ages. *J. Neurol. Neurosurg. Psychiat.* 1973. *36*. 455—466.

Castaigne, P., H. P. Cathala, L. Beaussart-Boulengé and M. Petrover: Effect of ischaemia on peripheral nerve function in patients with chronic renal failure undergoing dialysis treatment. *J. Neurol. Neurosurg. Psychiat.* 1972. *35*. 631—637.

Cathala, H. P. and J. Scherrer: Modifications du potentiel de nerf sous l'influence d'un brassard ischemiant chez l'homme. *Rev. Neurol.* 1963. *108*. 201—204.

Davis, H.: Some principles of sensory receptor action. *Physiol. Rev.* 1961. *41*. 391—416.

Fox J L and P J Kenmore The effect of ischemia on nerve conduction *Exp Neurol* 1967 *17* 403—419

Frank K and M G F Fuortes Excitation and conduction *Ann Rev Physiol* 1961 23 357—386

Kugelberg E Accomodation in human nerves *Acta physiol scand* 1944 Suppl 24 1—105

Lewis T G W Pickering and P Rothschild Centripetal paralysis arising out of arrested blood flow to the limb including notes on form of tingling *Heart* 1931 *16* 1—32

Lorente de No A study of nerve physiology *Stud Rockerfeller Inst med Res* 1947 *131* 1—496

Lorente de No R and G A Condouris Decremental conduction in peripheral nerve Integration of stimuli in the neuron *Proc nat Acad Sci* (Wash) 1959 *45* 592—617

Lundborg G Ischemic nerve injury Diss *Scand J Plast Reconstr Surg* 1970 Suppl 6 1—113

Magladery J W D B Dougal and J Stoll, Electrophysiological studies of nerve and reflex activity in normal man II The effects of peripheral ischemia *Bull Johns Hopk Hosp* 1950 *86* 291—312

Maruhashi J and E B Wright Effect of oxygen lack on the single isolated mammalian (rat) nerve fiber *J Neurophysiol* 1967 *30* 434—452

Nielsen V K Sensory and motor nerve conduction in the median nerve in normal subjects *Acta med scand* 1973 a *194* 435—443

Nielsen V K The peripheral nerve function in chronic renal failure V Sensory and motor conduction velocity *Acta med scand* 1973 b *194* 445—454

Nielsen V K The peripheral nerve function in chronic renal failure X Decremental nerve conduction in uremia? *Acta med scand* 1974 In press.

Nielsen V K and T Kardel Sensory nerve conduction during ischaemia in normal subjects and patients with chronic liver disease *Acta neurol scand* 1972 Suppl 51 465—469

Ochoa J T J Fowler and R W Gilliatt Anatomical changes in peripheral nerves compressed by a pneumatic tourniquet *J Anat* (Lond) 1972 *113* 433—455

Richards R L Ischaemic lesions of peripheral nerves A review *J Neurol Neurosurg Psychiat* 1951 *14* 76—87

Ruskin A P A Tanyag-Jocson and J B Rogoff Effects of ischemia on conduction of nerve fibers of varying diameter *Arch Phys Med* 1967 *48* 304—310

Shanes A M Electrochemical aspects of physiological and pharmacological action in excitable cells *Pharmacol Rev* 1958 *10* 59—73

Stenonis N (1667) Canis Carchariae dissectum caput. With english translation in Scherz, G (ed) Steno geological papers *Acta hist Sci nat med* (Kbh) 1969 20 88—90

Szpilman J and B Luchsinger, Zur Beziehung von Leitungs und Erregungsvermogen der Nervenfaser *Arch ges Physiol* 1881 *24* 347—357

Wright E B The effect of asphyxiation and narcosis on peripheral nerve polarization and conduction *Amer J Physiol* 1947 *148* 174—184

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From the Department of Histology[1] and Physiological Chemistry University of Umea Sweden

Effects of Alpha- and Beta-Receptor Blocking Agents on Catecholamine and 5-Hydroxytryptamine Induced Peroxidase and Amylase Secretion from Guinea Pig Submandibular Gland

By

Bengt Carlsoo[1] Ake Danielsson[1] Stefan Marklund[2] and Torgny Stigbrand[2]

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Abstract

Carlsoo B A Danielsson S Marklund and T Stigbrand *Effects of alpha and beta receptor blocking agents on catecholamine and 5 hydroxytryptamine induced peroxidase and amylase secretion from guinea pig submandibular gland* Acta physiol scand 1974 *92* 263—271

Enzyme secretion from incubated guinea pig submandibular gland slices was induced by adrenaline noradrenaline dopamine 5-hydroxytryptamine or dibutyryl cyclic AMP combined with theophylline Effects of alpha and beta blocking agents on stimulated peroxidase and amylase discharge were studied Propranolol (beta blocker) but not phenoxybenzamine (alpha blocker) markedly inhibited the secretion of both enzymes induced by adrenaline or noradrenaline Dopamine and 5-hydroxytryptamine were found to be potent enzyme secretagogues. Both propranolol and phenoxybenzamine antagonized the effects of these two amines The secretagogic effect of dibutyryl cyclic AMP in combination with theophylline was not affected by either propranolol or phenoxybenzamine L-DOPA and 5 hydroxytryptophan were without effect on enzyme secretion It is concluded that peroxidase and amylase are secreted in parallel from guinea pig submandibular gland and that enzyme release is mainly mediated via the beta adrenergic receptor

Salivary glands of several species are supplied with both alpha and beta adrenergic receptors (Emmelin 1967) The catecholamine induced amylase secretion from various mammalian salivary glands *in vivo* seems to be chiefly mediated via the beta adrenergic receptor (Pohto 1968 Katz and Mandel 1968 Yamamoto *et al* 1968) In the rat parotid gland this beta receptor activates enzyme secretion by enhancing the formation of cyclic AMP (Schramm and Naim 1970) We reported that peroxidase and amylase are simultaneously discharged from the guinea pig submandibular gland both *in vitro* and *in vivo* (Carlsoo *et al* 1972 1974 Bloom *et al* 1974) The regulation of amylase release from salivary glands has been extensively studied but little is known about the mechanisms regulating and governing peroxidase secretion from salivary glands

In the present study the effects of alpha and beta adrenergic blocking agents on catecholamine induced and 5 hydroxytryptamine induced peroxidase secretion from the guinea pig submandibular gland were investigated For comparison parallel measurements of amylase discharge were carried out

Material and Methods

Male guinea pigs three months of age weighing roughly 300 grams were used for the investigation The animals were starved overnight before being killed They were anesthetized by an intraperitoneal injection of sodium pentobarbital (Mebumal® ACO Sweden) The submandibular glands of both sides were rapidly excised and extraglandular tissues were removed under a stereomicroscope In each experiment the glands from two animals were used They were cut into small fragments which were randomly distributed among the incubation vessels All specimens were preincubated for 30 min in 3 ml of bicarbonate buffer (pH 7 4) supplemented with pyruvate glutamate and fumarate (Krebs 1950) and also containing albumin (5 mg/ml) and glucose (0 6 mg/ml) After preincubation the medium was replaced by 3 ml of fresh incubation medium containing the different secretagogues Control incubation without added substances were included in each experiment

The effects of phenoxybenzamine (alpha blocking agent) and propranolol (beta blocking agent) on enzyme secretion in response to noradrenaline adrenaline 5 hydroxytryptamine (5 HT) dopamine or N^{6}2 O dibutyryl cyclic adenosine monophosphate theophylline were tested The secretory effects of L-DOPA and 5 hydroxy L-tryptophan (5 HTP) were also investigated in the study The adrenergic blocking agents (phenoxybenzamine and propranolol) were present during both the preincubation and incubation periods

Preincubation as well as incubation were carried out at 37 C under continuous gassing with O_2—CO_2 (95 5) in a metabolic shaker (Danielsson 1974) After 60 min of incubation the specimens were removed by filtration through a nylon net and were homogenized in 3 ml of 50 mM phosphate buffer (pH 6 9) (Dahlqvist 1962) using an Ultra Turrax homogenizer (Janke und Kunkel K G Staufen Germany) run at a speed of 9 600 r p m for 45—60 s at 4 C The homogenates were centrifuged at about 3000 × *g* for 5 min The amylase activities in the crude homogenate and supernatant did not differ significantly Both incubation media and supernatants were assayed for peroxidase and amylase

Peroxidase assay Samples of tissue extract (supernatant) and incubation media were added to 16 7 mM pyrogallol in 200 mM sodium phosphate buffer (pH 6 0) in a total volume of 3 0 ml H_2O was added to a final concentration of 1 67 mM and the peroxidase activity was calculated from the initial rate of increase in absorbance at 400 nm Some of the test substances (5 HT and NA) apparently serve as substrates for lactoperoxidas (Carlsoo et al 1974) However the use of a high concentration of pyrogallol which has a very high reactivity with the peroxide compounds of lactoperoxidase eliminated any interference with the assay

The activities were expressed as $\triangle A_{400}$/min per g wet weight Peroxidase release was expressed as percentage of the total peroxidase activity in tissue and medium

Amylase assay Samples of tissue extracts (supernatants) or incubation media were appropriately diluted with 50 mM phosphate buffer and assayed for amylase using a micro-modification of the 3 5-dinitrosalicylate (DNS) method with 2 % soluble starch as substrate (Danielsson 1974) One unit of amylase was defined as the activity liberating reducing groups corresponding to 1 μmol of maltose monohydrate per min at 25 C The amylase release was expressed both as units/g tissue wet weight recorded in the medium and as the percentage of the total amylase activity in tissue and medium

Chemicals and drugs Soluble starch 3 5 dinitrosalicylic acid and pyrogallol were obtained from E Merck A G Darmstadt Germany All reagents were of analytical grade L-Noradrenaline bitartrate was purchased from Koch Light Laboratories Ltd Colnbrook Bucks England 5 Hydroxytryptamin creatinine sulfate (5 HT) L-3 4-dihydroxyphenylalanine (L-DOPA) 3 hydroxytryptamine HCl (Dopamine) 5 hydroxy L-tryptophan (5 HTP) and L-adrenaline bitartrate were purchased from Sigma Chemical Co St Louis Mo USA Theophylline was from Mann Research Laboratories Inc New York N Y USA N^{6}2 O dibutyryl adenosine 3 5-cyclic monophosphate was obtained from C F Boehringer und Soehne G m b H Mannheim Germany Propranolol (Inderal®) was from Imperial Chemical Industries Limited Alderly Park England and phenoxybenzamine hydrochloride from Smith Kline and French Labs Philadelphia USA

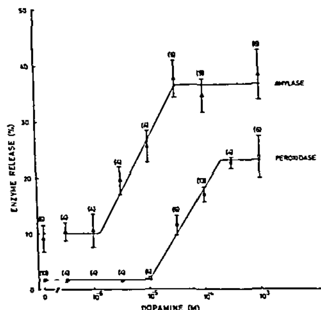


Fig 1 *Dose response curves for release of peroxidase and amylase by dopamine* Slices of guinea pig submandibular gland were preincubated at 37 C for 30 min in the supplemented Krebs bicarbonate buffer and then incubated for 60 min with dopamine The enzyme release is expressed as percentage of the total enzyme activity in tissue and medium Mean values (%) ± S E for indicated number of experiments (within brackets)

Statistical evaluation The statistical probability that the effect of an additive to the incubation medium was due to chance was estimated from the mean difference between the test and control incubations in a series of identical but separate experiments

Results

Fig 1 shows dose response curves for the stimulation of peroxidase and amylase secretion by dopamine At the plateau dopamine caused a discharge of 20 per cent peroxidase and 35 per cent amylase respectively The effects of phenoxybenzamine (alpha blocker 2×10^{-4} M) and propranolol (beta blocker 2×10^{-4} M) on peroxidase and amylase secretion induced by adrenaline noradrenaline dopamine 5 HT or DBcAMP combined with theophylline are shown in Table Ia and b Propranolol inhibited the secretory responses of all the tested amines However propranolol had no effect on enzymes discharge caused by DBcAMP theophylline Phenoxybenzamine was without effect on the adrenaline and noradrenaline induced peroxidase and amylase discharge whereas it markedly inhibited the secretory response to dopamine and 5 HT Table II shows the effects of lower concentrations of phenoxybenzamine (2×10^{-5} M) and propranolol (2×10^{-5} M) on dopamine and 5 HT induced enzyme secretion Also at these lower concentrations both adrenergic blocking agents significantly reduced the secretory response to dopamine and 5 HT However the alpha adrenergic blocker was less effective than the beta adrenergic blocker at this concentration

TABLE I a *In vitro effects of phenoxybenzamine and propranolol on the secretion of peroxidase from guinea pi* incubated in a supplemented Krebs bicarbonate buffer for 60 min with listed with theophylline The adrenergic blocking agents (2 × 10⁻⁴ M) were present during of the total peroxidase activity in tissue and medium Mean values (%) ± S E for

Secretagogues	No of exper	Non stimulated secretion	Control
NIL	5	–	1 1 ± 0 14
DBcAMP (1mM) + Theophylline (5 mM)	5	1 6 ± 0 14	10 7 ± 3 29
Noradrenaline (10⁻ M)	5	1 1 ± 0 17	12 3 ± 3 25
Adrenaline (10⁻⁴ M)	6	0 9 ± 0 10	6 6 ± 1 16
5 HT (10 M)	5	1 1 ± 0 17	6 8 ± 1 40
Dopamine (10⁻⁴ M)	5	2 8 ± 0 51	18 5 ± 3 20

P < 0 001
* P < 0 01
P < 0 05

L DOPA (10⁻⁴ M) and 5 hydroxytryptophan (10⁻⁴ M) were found to be without effect on both peroxidase and amylase discharge from the guinea pig submandibular gland

Control experiments revealed that the peroxidase secreted from the guinea pig submandibular gland was inactivated to about 80 per cent of the initially recorded

TABLE I b *In vitro effects of phenoxybenzamine and propranolol on the secretion of amylase from guinea pi* dase analyses (Table I a) The amylase release is expressed as percentage of the total

Secretagogues	No of exper	Non stimulated secretion	Control
NIL	5	–	6 8 ± 1 13
DBcAMP (1 mM) + Theophylline (5 mM)	5	14 0 ± 3 47	36 4 ± 6 08
Noradrenaline (10 M)	5	6 6 ± 1 70	37 6 ± 4 10
Adrenaline (10⁻ M)	6	10 3 ± 1 50	37 2 ± 3 77
5 HT (10⁻ M)	5	6 6 ± 1 70	20 6 ± 1 10
Dopamine (10⁻⁴ M)	5	16 0 ± 2 16	38 8 ± 7 74

* P < 0 001
P < 0 01
P < 0 05

submandibular gland After a preincubation period of 30 min at 37 C the specimens were concentrations of adrenaline noradrenaline dopamine 5 HT and DBcAMP in combination both the preincubation and incubation periods The enzyme release is expressed as percentage indicated number of experiments

Test substances		Difference control test	
α blocker Phenoxybenzamine (2×10⁻⁴ M)	β blocker Propranolol (2×10⁻ M)	α blocker	β blocker
1 3±0 22	0 9±0 09	+0 2±0 29	−0 2±0 18
19 8±2 29	13 5±6 55	+8 9±2 24	+2 8±2 27
10 0±2 12	0 8±0 22	−2 3±1 92	−11 5±3 06
5 9±0 90	0 7±0 30	−0 7±1 8?	−5 9±1 43
4 1±0 61	1 8±0 41	−? 8±0 93	−5 0±1 20
2 5±0 66	1 8±0 23	−16 0±3 65	−16 7±3 33

activity during the incubation period In the presence of 0 1 mM 5 HT the enzyme activity was fully protected Noradrenaline (0 1 mM) and dopamine (0 1 mM) prevented enzyme inactivation to about 80 per cent DBcAMP and theophylline showed no enzyme protecting capacity Amylase activity was not affected at all during the experimental procedure

submandibula gland The measurments were performed on th same samples as used for the peroxi amylase activity in tissue and medium Mean values ( )±S E

Test substances		Difference control test	
α blocker Phenoxybenzamine (2×10⁻ M)	β blocker Propranolol (2×10⁻ M)	α blocker	β blocker
9 3±3 12	5 3±1 38	+2 5±3 47	−1 5±1 16
40 4±4 86	33 8±4 40	+4 0±? 80	−2 6±4 67
30 9±4 01	13 6±4 40	−6 8±6 01	−?4 0±3 79
36 7±4 14	10 2±2 87	−0 5±? 46	−27 0±3 65
9 8±1 70	6 8±1 80	−10 8±1 7?	−13 8±0 82
12 4±2 30	13 4±1 76	− 5 4±6 27	−26 4±6 16

TABLE II *In vitro effects of low concentrations of phenoxybenzamine and propranolol on the secretion of peroxidase* in the presence of dopamine (10⁻ M) or 5 HT (10^{-4} M) for 60 min at 37 C The adre
Enzyme release is expressed as in Table I

Secretagogues	No of exper	Non stimulated secretion	Control
Amylase release			
5 HT (10^{-4} M)	6	6 8±0 81	24 9±5 55
Dopamine (10⁻ M)	6	6 8±0 81	4? 2±8 04
Peroxidase release			
5 HT (10⁻ M)	6	1 3±0 28	10 0±1 65
Dopamine (10 M)	6	1 3±0 28	15 5±2 47

[1] $P < 0.001$
[2] $P < 0.01$
[3] $P < 0.05$

Discussion

Amylase, a classical secretory digestive enzyme and main protein component of the parotid saliva is stored within zymogen granules of the glandular cells (Schramm and Danon 1961) Upon stimulation the granule bound enzyme is rapidly released from the acinar cells by exocytosis and is secreted into the saliva (Amsterdam et al 1969) Salivary glands of some mammalian species have also been shown to contain significant amounts of the oxidative enzyme lactoperoxidase (Alexander 1959 Morrison and Steele 1968 Thomson and Morell 1967 Kumlien 1972) and recent electron microscopic histochemical studies have revealed that this enzyme is also stored within the zymogen granules (e g Herzog and Miller 1970 Bloom et al 1974) Peroxidase positive material is secreted into the acinar lumina and ducts after *in vivo* stimulation of secretion (Bloom *et al* 1974)

In the present investigation as well as in previous *in vitro* studies (Carlsoo et al 1972 1974) a striking parallelism was noticed between the discharge of peroxidase and amylase in response to various secretagogues The adrenaline and noradrenaline induced secretion of both peroxidase and amylase was completely inhibited by the beta adrenergic blocking agent propranolol but was unaffected by the alpha blocker phenoxybenzamine These findings indicate that the catecholamine induced secretion of both enzymes from the guinea pig submandibular gland is mediated via a beta adrenergic receptor Neither propranolol nor phenoxybenzamine influenced the DBcAMP theophylline provoked enzyme discharge which excludes a non specific interference with the secretory process In addition the adrenergic blocking compound was found to be without effects of its own in eliciting enzyme discharge

Dopamine a precursor substance in the biosynthesis of adrenaline and noradrenaline was also found to be a potent secretagogue of both enzymes However

and amylase from guinea p g submandibular gland After preincubation the specimens were incubated nergic blocking agents (2×10⁻ M) were added to both the preincubation and incubation media

Test substances		Difference control test	
α blocker Phenoxybenzamine (2×10 M)	β blocker Propranolol (2×10⁻ M)	α blocker	β-blocker
18 4±4 94	12 2±2 95	−6.5±1 09	−12 5±3 70
24 5±4 31	11 4±1 59	−17 7±4 60	−30 8±6 65
6 7±1 30	4 0±0 43	− 3 3±1 14	−6 0±1 61
8 2±1 55	2 1±0 50	− 7 4±2 60	−13 4±2 12

the secretory effect of dopamine was inhibited not only by propranolol but also by phenoxybenzamine This difference in inhibitory pattern could be interpreted as indicating that there is a dopamine receptor in the guinea pig submandibular gland which is affected by both alpha and beta adrenergic blocking agents A specific dopamine receptor has recently been postulated to exist in the pancreas of the dog (Hashimoto *et al* 1971 Furuta *et al* 1973) and further in certain vascular areas the effect of dopamine has been *reported not to be mediated via adrenergic* receptors (Yeh *et al* 1969) Whether dopamine plays a physiological role in salivary gland function is not clear and ultimate proof of a specific dopamine receptor in these glands is still lacking

5 HT has previously been reported to stimulate enzyme secretion from salivary glands *in vitro* (Babad *et al* 1967) Furthermore fluid secretion from the abdominal salivary gland of the blowfly *Calliphora erythrocephala* is markedly increased by 5 HT (Berridge 1970) The 5-HT induced peroxidase and amylase discharge from the guinea pig submandibular gland is completely inhibited by the beta blocking agent propranolol as well as by the alpha blocking compound phenoxybenzamine Also in other secretory cell systems the effect of 5 HT has been reported to be inhibited by adrenergic blocking agents (Feldman and Lebovitz 1970) If 5 HT interacts with catecholamine receptors or if this indolamine exerts an effect of its own on a separate receptor in the guinea pig submandibular gland still remains to be elucidated It has recently been reported that 5 HT injected i v into rabbits markedly increased the amylase concentration of the secreted saliva and that the amylase secretory effect of 5 HT could be partly (45 per cent) inhibited by pretreatment of the animals with propranolol (Kojima *et al* 1973) The latter authors concluded that the 5 HT induced amylase secretion from the rabbit parotid gland *in vivo* is

the result of a combination of a direct action and an indirect one mediated via noradrenaline

Studies on amylase secretion from the rat parotid gland have shown that the catecholamines may play a dual role The amines cause enzyme discharge by acting on the beta receptors and by activating the alpha receptor they induce secretion of electrolytes and water (Schramm 1973) It has been suggested that the acinar cells of the rat parotid gland have two catecholamine receptors, one controlling enzyme secretion via the adenylate cyclase system (beta receptor) and the other regulating K release via a different pathway (alpha receptor) (Batzri *et al* 1971) K release mediated by the alpha receptor and associated with water transport may therefore facilitate the transport of the secretory proteins from the acinar lumina into the gland ducts (Batzri *et al* 1973)

In conclusion the oxidative enzyme peroxidase and the digestive enzyme amylase are not only simultaneously discharged from salivary glands but induction of their secretion by various secretagogues also seems to be mediated via the same receptor sites

References

ALEXANDER M N Iodide peroxidase in rat thyroid and salivary glands and its inhibition by antithyroid compounds *J biol Chem* 1959 *234* 1530—1533

AMSTERDAM A I OHAD and M SCHRAMM Dynamic changes in the ultrastructure of the acinar cell of the rat parotid gland during the secretory cycle *J cell Biol* 1969 *41* 753—773

BABAD H R. BEN ZVI A BOOLAH and M SCHRAMM The mechanism of enzyme secretion by the cell 4 Effects of inducers substrates and inhibitors on amylase secretion by rat parotid slices *Europ J Biochem* 1967 *1* 96—101

BATZRI S Z SELINGER and M SCHRAMM Potassium ion release and enzyme secretion adrenergic regulation by α and β receptors *Science* 1971 *174* 1029—1031

BATZRI S Z SELINGER M SCHRAMM and M R ROBINOVITCH Potassium release mediated by the epinephrine α receptor in rat parotid slices Properties and relation to enzyme secretion *J biol Chem* 1973 *248* 361—368

BERRIDGE M J The role of 5 hydroxytryptamine and cyclic AMP in the control of fluid secretion by isolated salivary glands *J Exp Biol* 1970 *53* 171—186

BLOOM G D B CARLSOO A DANIELSSON S MARKLUND and T STIGBRAND Peroxidase and amylase activity of the guinea pig submandibular gland during the secretory cycle *Histochemistry* 1974 *38* 271—280

CARLSOO B A DANIELSSON S MARKLUND and T STIGBRAND Simultaneous release of amylase and peroxidase from the guinea pig submandibular gland *FEBS Letters* 1972 *25* 69—72

CARLSOO B A DANIELSSON S MARKLUND and T STIGBRAND Effects of 3 5 -cyclic adenosine monophosphate 5 hydroxytryptamine noradrenaline and theophylline on the simultaneous release of peroxidase and amylase from the guinea pig submandibular gland *Acta physiol scand* 1974 *91* 203—210

DAHLQVIST A A method for the determination of amylase in intestinal content *Scand J clin Lab Invest* 1962 *14* 145—151

DANIELSSON A Techniques for measuring amylase secretion from pieces of mouse pancreas *Anal Biochem* 1974 *59* 220—234

EMMELIN N Nervous control of salivary glands *Handbook of Physiology* Vol II Ed Code Amer Physiol Soc Washington 1967 pp 595—632

FELDMAN J M and H E LEBOVITZ Mechanism of epinephrine and serotonin inhibition of insulin release in the golden hamster *in vitro Diabetes* 1970 *19* 480—486

FURUTA Y K HASHIMOTO K IWATSUKI and O TAKEUCHI Effects of enzyme inhibitors of catecholamine metabolism and of haloperidol on the pancreatic secretion induced by L-DOPA and by dopamine in dogs *Brit J Pharmacol* 1973 *47* 77—84

HASHIMOTO K. S SATOH and O TAKEUCHI Effect of dopamine on pancreatic secretion in the dog *Brit J Pharmacol* 1971 *43* 739—746
HERZOG V and F MILLER Die Lokalisation endogener Peroxydase in der Glandula parotis der Ratte *Z Zellforsch* 1970 *107* 403—420
KATZ R L and I D MANDEL Action and interaction of isoproterenol and alpha and beta adrenergic blocker on parotid and submaxillary secretions in man *Proc Soc exp Biol* (N Y) 1968 *128* 1140—1145
KOJIMA S M IKEDA and A TSUJIMOTO Effect of serotonin glucagon and other hormones on amylase secretion from rabbit parotid gland *Jap J Pharmacol* 1973 *23* 588—591
KREBS H A Body size and tissue respiration *Biochim biophys Acta* (Amst) 1950 *4* 249—269
KUMLIEN A Iodine metabolism and peroxidase activity in salivary glands *Acta endoc* (Kbh) 1972 *70* 239—246
MORRISON M and W F STEELE Lactoperoxidase the peroxidase in the salivary gland *Biology of the Mouth* Ed Person American Association for the Advancement of Science Washington D C 1968 pp 89—110
POHTO P Effect of isoprenaline pilocarpine and prenylamine on amylase secretion in rat parotid saliva *J oral Therap Pharmacol* 1968 *4* 467—474
SCHRAMM M The function of α and β adrenergic receptors and a cholinergic receptor in the secretory process in salivary gland cells *Cytopharmacology of Secretion an Advanced Study Institute* Venice and Milan 17—26 June 1973 p 36
SCHRAMM M and D DANON The mechanism of enzyme secretion by the cell I Storage of amylase in the zymogen granules of the rat parotis gland *Biochim biophys Acta* (Amst) 1961 *50* 102—112
SCHRAMM M and E NAIM Adenyl cyclase of rat parotid gland Activation by fluoride and norepinephrine *J biol Chem* 1970 *245* 3225—3231
THOMSON J and D B MORELL The structure location and distribution of salivary gland peroxidase *J Biochem* 1967 *62* 483—486
YAMAMOTO I R. INOKI and S KOJIMA Adrenergic receptors in amylase secretion from rabbit parotid gland *Europ J Pharmacol* 1968 *3* 123—130
YEH B K. J L McNAY and L I GOLDBERG Attenuation of dopamine renal and mesenteric vasodilation by haloperidol evidence for a specific dopamine receptor *J Pharmacol exp Ther* 1969 *168* 303—309

Acta physiol scand 1974 92 272—275
From the Department of Physiology University of Lund Sweden

Choline Acetyltransferase Activity in Rat Salivary Glands Enlarged by Isoprenaline Treatment

By

JORGEN EKSTROM

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Abstract

EKSTROM J *Choline acetyltransferase activity in rat salivary glands enlarged by isoprenaline treatment* Acta physiol scand 1974 92 272—275

Certain procedures have earlier been found to cause the weight of the rat parotid gland to vary After these procedures the choline acetyltransferase activity in the gland was also found to have changed in the same direction as the weight. This might suggest that variations in the gland mass in itself could influence the choline acetyltransferase activity However the present study shows that this does not seem to be the case since a marked enlargement of the parotid gland, induced by isoprenaline treatment can occur without being accompanied by any increase in the activity of this enzyme

The acetylcholine synthesizing enzyme choline acetyltransferase is in salivary glands localized in the cholinergic nerves (Nordenfelt 1963 Ekstrom and Holmberg 1972) In the rat parotid gland the activity of this enzyme has been found to decrease when the rats are fed on a liquid diet and to increase on a dry cellulose rich diet or when dryness of the oral mucosa is produced by atropinization the changes in enzyme activity are thought to have been caused by a decrease and an increase respectively in the reflexly elicited traffic of secretory impulses in the parasympathetic neurones to the gland (Ekstrom 1973 1974) However since the gland weight varied in a similar way as the choline acetyltransferase activity it is possible that a change of the gland mass in itself might influence the activity of this enzyme such a possibility was also considered in a recent study on the changes of the skeletal muscle mass of the rat (Diamond Franklin and Milfay 1974) Isoprenaline given repeatedly is known to cause a marked gain in the weight of the parotid gland and this is considered to depend on a direct action of the substance on the gland cells (see Potho 1966 Seifert 1967 Emmelin Schneyer and Schneyer 1973)

Key words Autonomic nervous system —salivary glands—isoprenaline—choline acetyltransferase activity

In the present study choline acetyltransferase activity was estimated in rat salivary glands after prolonged treatment with isoprenaline in order to see if increase in gland size as such may affect the capacity to synthesize acetylcholine in the neurones of the glands

Methods

Female rats 3 to 4 months old and weighing about 200 g of a Sprague Dawley strain bred at this Institute were used The rats were given a standard pelleted diet and water *ad libitum* Isoprenaline sulphate 3 mg in 0 2 ml saline was given s c every 8 h for 21 days to 10 rats as controls served 10 corresponding litter mates In preliminary acute experiments rats were anesthetized with chloralose and fine glass cannulae were inserted into the ducts of the parotid and the submaxillary glands The dose of isoprenaline used evoked a slow flow of saliva from the submaxillary gland which continued for about 8 h The secretion from the parotid gland was very scanty and stopped much earlier The amount of secreted saliva was about ten times less from the parotid than from the submaxillary gland

The rats were killed with ether 12—20 h after the last injection of isoprenaline The major salivary glands were then carefully taken out cleaned washed in saline and weighed Each pair of glands of individual rats was pooled except for the sublingual glands where the glands of two rats were pooled The acetone dried powder of the gland was made up in cysteine saline in a concentration of 50 mg/ml (parotid and submaxillary) or 25 mg/ml (sublingual) The activity of choline acetyltransferase was determined according to the method of Hebb (see Nordenfelt 1963 1965) Tissue extracts 0 2 or 0 4 ml for det glands of the isoprenaline treated rat 0 2 ml for the glands of the untreated rat were incubated for 1 h at 38 C The acetyl choline formed was estimated on the frog rectus and expressed in μg acetylcholine chloride formed per h per pool of whole glands (total activity) and in μg acetylcholine chloride formed per h per g acetone powder (concentration) For the statistical analysis Student s t test was used paired comparisons were made between the drug treated rat and its untreated litter mate

Results

Neither at the start nor at the end of the experimental period were there any differences in body weights between the two groups of rats

Gland weights As seen from Table I all the gland increased significantly ($p < 0.001$) after isoprenaline treatment the parotid 10 fold the submaxillary 5 fold and the sublingual by about 15 % The dry weights (acetone dried powders) were affected in a similar way as the wet weights

Choline acetyltransferase activity The table shows that the total enzyme activity did not change in the parotid and the sublingual glands after isoprenaline treatment In the submaxillary gland a reduction of the total activity by about 37 % was found ($p < 0.001$) Due to the gland enlargements and in the case of the submaxillary gland also to the decrease in total enzyme activity the concentration of the enzyme is lowered in the glands of the isoprenaline treated rats

Discussion

As previously shown by Schneyer (1962) all the major salivary glands in the rat are found to increase in size after isoprenaline treatment and the parotid most and the sublingual least The enlargement is thought to be mediated via beta adrenergic

TABLE I Weight and choline acetyltransferase activity of salivary glands in untreated rats and in rats treated with isoprenaline for 21 days Values are mean ± S E

Glands		Wet weights (mg)	Enzyme activity in μg ACh/h/pooled glands	Enzyme activity in μg ACh/h/g acetone powder
Parotid	Isoprenaline	1482.8 ± 96.5 (n = 20)[3]	49.5 ± 3.4 (n = 10)[1]	78 ± 6 (n = 10)
	Control	140.2 ± 5.4 (n = 20)	51.4 ± 3.8 (n = 10)	835 ± 44 (n = 10)
Submaxillary	Isoprenaline	863.5 ± 66.8 (n = 20)[3]	28.7 ± 2.4 (n = 9)[2]	84 ± 18 (n = 9)
	Control	169.5 ± 5.6 (n = 20)	45.7 ± 4.0 (n = 9)	702 ± 15 (n = 9)
Sublingual	Isoprenaline	44.6 ± 1.1 (n = 20)	24.3 ± 1.7 (n = 5)[1]	816 ± 47 (n = 5)[2]
	Control	38.7 ± 0.8 (n = 20)	24.7 ± 1.3 (n = 5)	1054 ± 95 (n = 5)

[1] > 0.1 — [2] < 0.05 — [3] < 0.001 when compared with corresponding Control

receptors (see Emmelin *et al* 1973) Earlier studies (Ohlin 1966 a Schneyer 1969) suggest that the gain in weight cannot solely reflect increased secretory activity, this is also illustrated in the present investigation by the far less amount of saliva secreted from the parotid than from the submaxillary gland after a single injection of isoprenaline

The present study shows that a very big increase of the parotid gland mass can occur without a concomitant increase of the total choline acetyltransferase activity This result suggests that the enhancement of the enzyme activity which was demonstrated in enlarged parotid glands in a previous investigation (Ekstrom 1974) probably occurred independently of the weight increase and not as a consequence of it the likely cause of the enhanced enzyme activity seems to be increased flow of secretory impulses in the parasympathetic nerves to the gland It has earlier been shown (Ohlin and Perec 1967 a) that the choline acetyltransferase activity of the submaxillary gland does not decrease when a fall in gland weight is produced by duct ligation

Contrary to the 2 other glands the submaxillary responded to the isoprenaline treatment with a marked decrease in the transferase activity There is no ready explanation of this observation The fall in enzyme activity was of the same order of magnitude as that found in the gland after parasympathetic decentralization (Ohlin and Perec 1967 b) It may be worth mentioning that isoprenaline treatment has another effect on this gland also seen after section of the preganglionic parasympathetic neurones it caused a supersensitivity to secretory drugs (Ohlin 1965 1966 b) Of interest may also be to note that the glandular cells of the submaxillary gland are much better supplied with adrenergic fibres than the other glands (Norberg and Olsson 1965) and furthermore to combine the present finding that the enzyme activity is reduced after prolonged action of a sympathomimetic drug with the observation that the enzyme activity increases in this particular but not in the other glands after removal of adrenergic impulses by surgical or chemical sympathectomy (Nordenfelt 1964 Ekstrom 1972)

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References

DIAMOND I G M FRANKLIN and D MILFAY The relationship of choline acetyltransferase activity at the neuromuscular junction to changes in muscle mass and function *J Physiol* (Lond) 1974 *236* 247—257

EKSTROM J Choline acetyltransferase in salivary glands after surgical and chemical sympathectomy *Acta physiol scand* 1972 *86* 539—545

EKSTROM J Choline acetyltransferase and secretory responses of the rat s salivary glands after liquid diet *Quart J exp Physiol* 1973 *58* 171—179

EKSTROM J Choline acetyltransferase activity in rat salivary glands after cellulose rich diet or treatment with an atropine like drug *Quart J exp Physiol* 1974 *59* 191—199

EKSTROM J and J HOLMBERG Choline acetyltransferase in the normal and parasympathetically denervated parotid gland of the dog *Acta physiol scand* 1972 *86* 353—358

EMMELIN N C A SCHNEYER and L H SCHNEYER The pharmacology of salivary secretion In *International Encyclopedia of Pharmacology and Therapeutics* Sec 39 A Vol I Ed P Holton Pergamon Oxford and New York 1973 1—39

NORBERG K A and L OLSON Adrenergic innervation of the salivary glands in the rat *Z Zellforsch* 1965 *68* 183—189

NORDENFELT I Choline acetylase in normal and denervated salivary glands *Quart J exp Physiol* 1963 *48* 67—79

NORDENFELT I Choline acetylase in salivary glands of the rabbit dog and rat after sympathetic denervation *Acta Univ Lund* 1964 Sectio II No 10 1—7

NORDENFELT I *Acetylcholine synthesis in salivary glands* Hakan Ohlsson Lund 1965 (Thesis)

OHLIN P Secretory responses of innervated and denervated submaxillary glands of rats *Acta Univ Lund* 1965 Sectio II No 23 1—12

OHLIN P Effects of treatment with isoprenaline or pilocarpine on the secretory responses of the parasympathetically denervated submaxillary gland of the rat *J Oral Ther Pharmacol* 1966 a *2* 376—379

OHLIN P Effects of isoprenaline treatment on secretory responses and respiratory enzymes of the submaxillary gland of the rat *J Oral Ther Pharmacol* 1966 b *3* 190—193

OHLIN P and C PEREC Secretory responses and choline acetylase of the rat s submaxillary gland after duct ligation *Experientia* (Basel) 1967 a *23* 248—249

OHLIN P and C PEREC Choline acetylase in normal and denervated submaxillary glands of rats after repeated teeth amputations *Acta physiol scand* 1967 b *69* 134—139

POHTO P Catecholamine induced salivary gland enlargement in rats *Acta odont scand* 1966 *24* Suppl 45 (Thesis)

SCHNEYER, C A Salivary gland changes after isoproterenol induced enlargement *Amer J Physiol* 1962 *203* 232—236

SCHNEYER, C A β adrenergic effects by autonomic agents on mitosis and hypertrophy in rat parotid *Proc Soc exp Biol* (NY) 1969 *131* 71—75

SEIFERT G Experimental sialadenosis by isoproterenol and other agents histochemistry and electron microscopy In *Secretory mechanisms of salivary glands* Eds L H Schneyer and C A Schneyer Academic Press New York and London 1967 191—208

Acta physiol scand 1974 92 276—282

From the Department of Physiology Karolinska Institutet Stockholm Sweden

Effects of Sympathetic Stimulation on Facial Muscle Contractions

By

CHRISTER LINDQUIST

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Abstract

LINDQUIST CHR *Effects of sympathetic stimulation on facial muscle contractions* Acta physiol scand. 1974 *92* 276—282

Low frequency stimulation (1—10 Hz) of the cervical sympathetic nerve is shown to increase twitch tension in the non fatigued orbicularis oculi and oris muscles by up to 15 % and to increase contraction and half relaxation times The eff ct sets in 15—45 s following start of sympathetic stimulation reaches a peak in 50—100 s and outlasts the stimulation by up to 90 s The potentiating effect is not accompanied by any signs of changes in neuromuscular transmission or conduction. It is blocked by the β blocker propranolol The mechanism of sympathetic action is discussed as well as its possible physiological significance

The possible presence of non twitch fibers of the slow tonic type in facial muscles is tested by i v injections of suxamethonium and decamethonium. Even relatively small doses not affecting neuromuscular transmission may produce long lasting contractions in the orbicularis oculi and oris It is concluded that these contractions suggest specific properties of the twitch fibers rather than pointing to the presence of slow tonic muscle fibers

From a number of investigations it is well known that sympathomimetic amines affect contractions of striated muscles when injected directly into the blood stream in relatively large amounts Whether activity in the sympathetic muscle nerves changes the performance of these muscles is more doubtful The contractile force of fatigued skeletal muscles may increase in response to electrical stimulation of the sympathetic chain but the stimulus frequencies required exceed those considered to correspond to physiological discharge rates in vasomotor nerve fibers (*cf* Folkow 1952) On sympathetic stimulation at physiological frequencies no contractile effects whatever have been recorded from skeletal muscles whether fatigued or not The question has therefore arisen that the effects of high frequency stimulation should reflect some function of the sympathetic nerves other than that of vasomotor regulation

This paper will present results from studies of the effects of sympathetic stimulation on facial muscle contractions As a consequence of these results it may be worth while to reconsider the functional role of the sympathetic innervation of striated

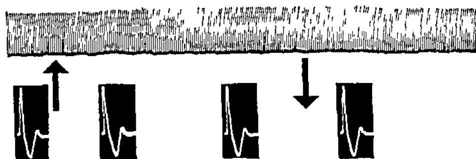


Fig 1 Potentiating effect of sympathetic stimulation on orbicularis oris twitch contractions (upper record) Stimulation period between arrows is 130 s The muscle compound action potential is unaffected (lower records) Full description in text

muscles The facial muscles were chosen for the experiments because their contraction properties are well known from recent studies (Lindquist 1973) and because their sympathetic innervation is derived from the cervical sympathetic which can easily be prepared for selective electrical stimulation Effects on twitch contractions were recorded both from the orbicularis oris and the orbicularis oculi but this report will be limited to the former muscle since studies of the latter were hampered by contractions of the smooth muscles in the orbit elicited by the sympathetic stimulation

As will be shown stimulation of the sympathetic nerve at physiological frequencies enhances and prolongs twitch contractions of the orbicularis oculi and oris The effects can be reproduced by i v injections of adrenaline and noradrenaline and are reduced by a β adrenergic blocking drug A slow increase in facial muscle tone sometimes observed on sympathetic stimulation raised the question whether these muscles have any non twitch fibers of the slow tonic type This was tested by injections of suxamethonium and decamethonium the results will be presented and discussed below

Methods

10 cats (2 5—3 5 kg) were used for the experiments Anesthesia was induced by i p injections of Nembutal® 40 mg per kg b wt and maintained by additional i v injections A Grass FT 03 force-displacement transducer was used for r cording facial musle contractions Musle action potentials were recorded by DISA bipolar concentric needle electrodes The surgical procedures and the recording and stimulating equipment used have been described previously (Lindq ist 1973)

Results

Typical changes in the twitch contractions of the orbicularis oris on stimulation of the cervical sympathetic nerve are illustrated in the upper tracing of Fig 1 the lower tracing shows gross action potentials recorded from the muscle The

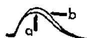


Fig 2



Fig 3

Fig 2 Superimposed records of orbicularis oris contraction curves without (a) and during (b) sympathetic stimulation Full description in text

Fig 3 Contractile responses of orbicularis oris to i v injections of suxamethonium (Celocurin®) in doses of 25 μg/kg b wt (A) 50 μg/kg (B) and 100 μg/kg Time bar 60 s Full description in text

muscle nerve is stimulated supramaximally at a frequency of 1 Hz until a twitch contraction of stable amplitude is obtained The muscle is then in a non fatigued state (*cf* Edstrom and Lindquist 1973) At the arrow pointing upwards sympathetic stimulation starts In this case the stimulus strength is 10 V the stimulus duration 0 3 ms and the frequency 6 Hz A gradual increase in twitch tension sets in 20 s following onset of stimulation and after 50 s the twitch potentiation attains its maximum At this frequency the maximal twitch is 15 % stronger than the value before the stimulation In this experiment a potentiation of twitch tension was observed even at 1 Hz At a frequency of 2 Hz the tension was 6 % higher at 4 Hz 7 % and at 10 Hz 15 % higher than the prestimulus value On cessation of stimulation (at the arrow pointing downwards) the potentiation is maintained for 60 s Throughout the period of stimulation there is no change in the continuously monitored gross muscle action potential The sympathetic stimulation should thus affect the contractile elements rather than the impulse transmission or conduction Fig 2 from another experiment, shows that the potentiation of twitch tension is typically accompanied by changes in the time course of the individual twitches as well The curve marked *a* is obtained before onset of sympathetic stimulation and the curve marked *b* when the stimulation has been going on for 3 min In addition to a small twitch potentiation there is a prolongation of the contraction time and the relaxation phase in the course of the period of stimulation

It was a general finding that within a frequency range of up to 10 Hz the twitch tension potentiation increased with the frequency of the stimulation applied to the cervical sympathetic In most experiments the mean of maximum potentiation was around 10 % but in a few experiments no sympathetic effects whatever could be detected The effect of sympathetic stimulation was also tested on fatigued muscles (*cf* Edstrom and Lindquist 1973) with similar results Effects were obtained even at low stimulus frequencies which is of particular interest since the normal working range of impulse frequencies in *e g* sympathetic vasoconstrictor fibres seems to be 1—10 Hz (Folkow 1952)

In 3 expts in which the time relations of the sympathetic effects were studied more closely their latency was found to be 15—45 s the maximal effects being reached in 50—100 s The slow onset seems to fit well with the concept of a metabolic mechanism of action (*cf* Discussion) In this context it may be worth noting that



Fig 4 Increase in orbicularis oculi background tone in response to i v injections of 2 μg (A) and 10 μg (B) decamethonium (Syncurine®) per kg b wt without appreciable effect on maximal twitch contractions Time bars 20 s Full description in text

staircase effects have a time course not unlike that of the sympathetic effects (*cf* Edstrom and Lindquist 1973)

At a stimulus duration of 0 3 ms the thresholds of the sympathetic effects were 5—10 V In 2 expts these thresholds were compared to those of other sympathetic responses in the face watched through a dissecting microscope Piloerection in the facial skin and withdrawal of the nictitating membrane were found to occur at 0 4 V and vasoconstriction in the lower lip at 4 V The significance of the differences in threshold between the sympathetic effects on facial muscle contractions and other sympathetic responses will be considered in the Discussion

Sympathetic stimulation may activate cholinergic vasodilator fibers supplying facial muscles (*cf* Euler and Gaddum 1931) in addition to adrenergic vasocon strictor fibers The sympathetic effects described above involve adrenergic trans mission since they are reproduced by injections of noradrenaline and adrenaline (< 10 μg/kg body weight i v) However they do not seem to be caused by vaso-constriction since 1) clamping the carotid arteries does not result in a twitch tension increase 2) there is a threshold difference between the increases in twitch tension and the visible vasoconstriction in the lip and 3) twitch potentiation persists fol lowing injections of α blocking drugs (*cf* below)

The type of adrenergic receptors mediating the increments in twitch tension was studied by blocking α receptors with phentolamine (Regitin®) and β receptors with propranolol (Inderal®) α blockade did not noticeably alter the studied effects but following β blockade they were drastically reduced Thus potentiation of twitch tension by sympathetic stimulation should involve activation of adrenergic β receptors

During the experiments reported above a slowly developing increase in tone of the facial muscle was sometimes noted when the sympathetic stimulation was started This increase in tone proved to occur also if the cervical sympathetic nerve was stimulated without concomitant muscle nerve stimulation The latency of the response was only 2—3 s In the orbicularis oris the contraction amounted to 5—10 % of a maximal twitch but in the orbicularis oculi it was often stronger It was blocked by the α blocker phentolamine The denervated in contrast to the normally innervated facial and tongue muscles have previously been found to yield contractile responses to stimulation of the cervical sympathetic nerve (Euler and Gaddum

1931) The thresholds of the responses recorded in the present series of experiments were in the same range a those of piloerection in the facial skin or contractions of the nictitating membrane and it proved extremely difficult to rule out that contractions from the smooth muscles involved in these responses were transmitted to the recording system In this context attention must however be drawn to the fact that the cat s extraocular muscles contract in response to sympathetic stimulation Since these responses have been presumed to be set up in slow tonic muscle fibers (Eakins and Katz 1967) experiments were also performed to test whether the facial muscles may contain such a fiber system

Testing for slow tonic fibers In contrast to ordinary twitch fibers the slow tonic muscle fibers develop a long lasting contracture in response to depolarizing solutions (*cf* Kuffler and Vaughan Williams 1953) Suxamethonium (Celocurin®) and decamethonium (Syncurine®) injections were therefore used as a convenient way of testing for slow tonic fibers in the facial muscles The outcome of such an experiment is illustrated in Fig 3 The response of the orbicularis oris is recorded from the angle of the mouth In *A* injection of suxamethonium in a dose of 25 μg/kg b wt elicits a contraction with a peak amplitude amounting to 8 % of a maximal twitch response and of a duration of approximately 60 s In *B* following injection of 50 μg/kg the response is 20 % of the maximal twitch and the duration 100 s In *C* finally after an injection of 100 μg/kg the response is 50 % of the maximal twitch and the duration 5 min

In Fig 4 maximal twitches of the orbicularis oculi are elicited at a frequency of 1 Hz after i v injection of decamethonium in doses of 2 μg/kg (A) and 10 μg/kg (B) respectively A contractile response similar to that in Fig 3 is recorded without any appreciable depression of the twitch contraction On repeated drug injections marked tachyphylaxis developed The behavior of the facial muscles resembles that of extraocular muscles (Katz and Eakins 1966) In contrast to previous experimental results (Lindquist 1973) the findings reported here may thus indicate that the cat s facial muscles do in fact contain tonic fibers However it may well be that th s kind of reaction to depolarizing curare agents is not always due to any particular contraction properties (*cf* Discussion)

Discussion

The effects of sympathomimetic amines on striated extremity muscles have been extensively studied (for review see Bowman and Nott 1969), *it is known that twitch* potentiation may be accomplished by four different mechanisms *1) by a lowering of* the threshold for electrical excitation in the muscle nerve (Bülbring and Whitteridge 1941) 2) by an increase in the amount of acetylcholine liberated in the motor nerve endings (Krnjevic and Miledi 1958) 3) by changes in the membrane potential of the muscle fibers (Bowman and Nott 1969) and 4) by activation of the adenyl-cyclase 3 5 AMP system (Bowman and Nott 1969)

In the experiments reported here, alternative 1 cannot be applicable since supramaximal muscle nerve stimulation was always used. As far as the other three alternatives are concerned only the latter two mechanisms are blocked by β receptor blocking drugs as are the sympathetic effects on the facial musles. A change in the muscle fiber membrane potential (alternative 3) should be reflected in the gross muscle action potential. Since however no changes in this potential occurred on sympathetic stimulation of the facial muscles the only possible alternative that might account for the twitch potentiation observed in the experiments should be the fourth, i.e. the metabolic action of adrenergic substances. Such a relatively slow mechanism might also account for the slow onset of the sympathetic effects and explain why the twitch potentiation outlasted the sympathetic stimulation (cf. above).

Where is the sympathetic transmittor released? In extremity muscles all adrenergic terminals are found in close proximity to blood vessels (Fuxe and Sedvall 1965). It is possible that noradrenaline released at vasoconstrictor nerve endings may diffuse to and affect the striated muscle fibers as has been suggested (cf. Bowman and Nott 1969). The thresholds for the sympathetic twitch potentiating effects were however higher than those observed for vasoconstriction in the lip. Unless this indicates a difference in threshold between vasoconstrictors to the lip and those to the underlying muscles, it may mean that impulses causing changes in the muscle contractions are conveyed in a particular group of sympathetic efferents. This group of fibers could possibly be identical with the fourth group of sympathetic fibers described by Bishop and Heinbecker (1932), which had the highest threshold for electrical stimulation and the stimulation of which did not produce any of the well known sympathetic responses in the face.

By means of electron microscopy Santini and Ibata (1971) were recently able to show that some unmyelinated sympathetic nerve filaments supply intrafusal muscle fibers in the cat. They also claim that they have found a direct sympathetic innervation of extrafusal muscle fibers as well. In order to test whether adrenergic terminals do actually occur in the facial musculature, fluorescence microscopy was applied in some experiments (Hökfelt and Lindquist unpublished observations). Such adrenergic terminals could be unequivocally demonstrated only in connection with blood vessels and it is possible that a direct innervation of the muscle fibers, if present, can be detected only by means of electron microscopy.

Do so called slow tonic non twitch fibers occur in the facial muscles? Injections of suxamethonium or decamethonium yield contractile responses of appreciable amplitude. If some part of such a long lasting contraction should be attributable to activity in slow tonic fibers it ought to be possible to record a tetanic contraction from these fibers. Since the high fusion frequencies of the facial muscles (Edstrom and Lindquist 1973) are in marked contrast to the low fusion frequencies previously recorded in slow tonic fibers (Hess and Pilar 1963) it should not be difficult to distinguish between responses from these fibers and such from conventional muscle fibers even though recordings are made from the entire muscle. In a previous investigation of the contraction properties of the facial muscles (Lindquist 1973)

no evidence in support of the presence of slow tonic fibers could be found in spite of the apparently favorable experimental conditions Thus the question arises whether depolarizing curare agents may be able to set up long lasting contractions also in certain twitch fibers Similar ideas have previously been advanced in connection with experimental results obtained in studies of the middle ear muscles in cat and rabbit (Wersall 1958) Strong longlasting contractile responses have also been recorded from m rectus superior in cat on injection of succinylcholine and decamethonium (e g 10 g tension 2 min after injection of 128 μg succinylcholine per kg b wt see Katz and Eakins 1966 Fig 2) The responses in the extraocular muscles have been attributed to slow tonic muscle fibers In recent investigations on cat and monkey (Fuchs and Luschei 1971) it could however be demonstrated that even on tetanic contraction slow tonic fibers do not noticeably contribute to the contractile response set up in extraocular muscles on stimulation of their muscle nerves There is thus reason to believe that also the long lasting contractions evoked in extraocular muscles following injections of suxamethonium and decamethonium should to a great extent be due to activity in the ordinary twitch fibers

References

Bishop G H and P Heinbecker A functional analysis of the cervical sympathetic nerve supply to the eye *Amer J Physiol* 1932 *100* 519—532

Bowman W C and M W Nott Actions of sympathomimetic amines and their antagonists on skeletal muscle *Pharmacol Rev* 1969 *21* 27—72

Bulbring E and D Whitteridge The effect of adrenaline on nerve action potentials *J Physiol* (Lond) 1941 *99* 201—207

Eakins K E and R L Katz The effects of sympathetic stimulation and epinephrine on the superior rectus muscle of the cat *J Pharmacol exp Ther* 1967 *157* 524—531

Edstrom L and Chr. Lindquist Histochemical fiber composition of some facial muscles in the cat in relation to their contraction properties *Acta physiol scand* 1973 *89* 491—503

Euler, U S v and J H Gaddum Pseudomotor contractures after degeneration of the facial nerve *J Physiol* (Lond) 1931 *73* 54—66

Folkow B Impulse frequency in sympathetic vasomotor fibres correlated to the release and elimination of the transmittor *Acta physiol scand* 1952 *25* 49—76

Fuchs A F and E S Luschei Development of isometric tension in simian extraocular muscl *J Physiol* (Lond) 1971 *219* 155—166

Fuxe K and G Sedvall The distribution of adrenergic nerve fibres to the blood vessels in skeletal muscle *Acta physiol scand* 1965 *64* 75—86

Hess A and G Pilar Slow fibres in the extraocular muscles of the cat *J Physiol* (Lond) 1963 *169* 780—798

Katz R L and K E Eakins The effects of succinylcholine decamethonium hexacarbacholine gallamine and dimethyl tubocurarine on the twitch and tonic neuromuscular systems of the cat *J Pharmacol exp Ther* 1966 *154* 303—309

Krnjevic K and R Miledi Some effects produced by adrenaline upon neuromuscular propagation *J Physiol* (Lond) 1958 *141* 291—304

Kuffler S W and E M Vaughan Williams Properties of the slow' skeletal muscles fibres of the frog *J Physiol* (Lond) 1953 *121* 318—340

Lindquist Chr Contraction properties of cat facial muscles *Acta physiol scand* 1973 *89* 482—490

Santini M and Y Ibata The fine structure on thin unmyelinated axons within muscles spindles *Brain Res* 1971 *33* 289—302

Wersall R The tympanic muscles and their reflexes *Acta oto-laryng* (Stockh) 1958 Suppl 139

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From the Department of Physiology I Karolinska Institutet Stockholm Sweden

Phenoxybenzamine Blockade of the Enhancing Effect of Substance P on the Twitch Induced by Transmural Nerve Stimulation in the Guinea Pig Vas Deferens

By

U S VON EULER and P HEDQVIST

Synthetic Substance P (SP) (Tregear *et al* 1971) like a number of other smooth muscle stimulating compounds including acetylcholine (ACh) enhances the twitch response of the isolated guinea pig vas deferens elicited by field stimulation of its postganglionic nerves (Sjostrand and Swedin 1968 Euler and Hedqvist 1974) As previously reported phenoxybenzamine (PBA) leaves the twitch unaffected (for references see Ambache and Zar 1971)

The present report describes the annulment of the action of SP on the twitch respon e when the isolated guinea pig vas deferens is exposed to PBA in a concentra tion of 1 μg/ml for a period of 1/2—2 h The effect of this treatment on the action of other drugs on the twitch will also be reported

The isolated guinea pig vas deferens was mounted in a 5 ml bath with Tyrode as bath flu d (for composition see Euler and Hedqvist 1972) gassed with 95 % O and 5 % CO_2 Transmural stimuli were delivered by a Grass stimulator through platinum wires along the wall of the bath Twitches were elicited at 1 min intervals by 25 pulses at 5 or 10 Hz pulse duration 1 ms supramaximal voltage The contractions were recorded by an isotonic transducer on a Honeywell ink writer The load on the organ was 0 25—0 5 g The following drugs were used Synthetic SP (kindly given to us by Prof S Leeman Cambridge Mass U S A) Acetylcholine chloride ( )noradrenaline bitartrate (NA) phenoxybenzamine hydrochloride phentolamine hydrochloride potassium chloride prostaglandin E (PGE)

After recording the enhancing effects of SP ACh and KCl as ell as the inhibitory actions of PGE and NA on the nerve induced twitch with ordinary Tyrode in the bath the medium was replaced by Tyrode containing 1 μg PBA per ml (PBA Tyrode)

After 10—30 min in PBA Tyrode the previous enhancing effect of 0 1 μg/ml ACh on the twitch was abolished At this time the effect of SP vas markedly inhibited whereas the action of KCl (0 4 mg/ml) was much less influenced (Fig 1) The in hibitory action of NA (0 4 μg/ml) was somewhat weakened

After 1—2 h with PBA Tyrode when the twitches were largely unchanged the enhancing action of SP (2—6 ng/ml) was abolished No significant alteration in the enhancing effect of KCl was however noticed (Fig 1) At this time the inhibitory effect of NA vas further diminished whereas that of PGE remained unchanged After 1 h exposure to phentolamine (1 μg/ml) the enhancing action of SP on the twitch was only moderately reduced

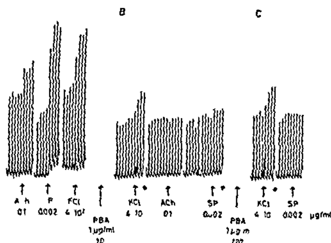


Fig 1 Isolated guinea pig vas deferens in 5 ml Tyrod at 37 transmurally stimulated once every minute 25 pulses 10 Hz 1 ms A Effects of acetylcholine (ACh) Substance P (SP) and KCl on twitch responses in ordinary Tyrode B and C 10 and 120 min respectively after addition of 1 µg/ml phenoxybenzamine (PBA) to Tyrode solution Drugs added as indicated

Whereas PBA thus abolished the enhancing effect of SP on the twitch response it did not inhibit the direct stimulating action of SP on the vas (Fig 2) not even after exposure of the organ to the drug for 2 h On the other hand the direct stimulating effect of ACh was rapidly blocked by PBA The direct effect of KCl was either unaffected or moderately increased after addition of PBA to the Tyrode bath Increased responses to SP after PBA were also observed in several cases (*cf* Fig 2)

The annulment of the enhancing effect of SP on the twitch but not the direct contracting action by prolonged treatment with PBA suggests that SP affects the twitch by an action different from that which contracts the smooth muscle directly

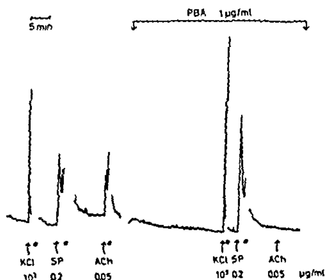


Fig 2 Isolated guinea pig vas deferens in 5 ml Tyrode at 37 Addition of KCl Substance P (SP) and acetylcholine (ACh) to preparation in ordinary Tyrode and after addition of phenoxybenzamine (PBA) 1 µg/ml

It also implies that PBA in the concentration used blocks a prejunctional effect of SP As expected the treatment with PBA also blocked the direct contracting effect of NA on the vas deferens The blocking effect of PBA on the effect of ACh on the vas is in agreement with its known cholinolytic action

Ambache and Zar (1971) have pointed out some facts speaking against NA as the chemical transmitter in the guinea pig vas deferens Furness and Iwayama (1972) on the other hand seek to explain the failure of adrenergic blockers to annul the twitch by assuming that the drug does not reach the NA receptors in effective concentrations Our results seem to support the opinion of Ambache and Zar (1971) as far as the twitch is concerned since it is hard to imagine that PBA should not be able to affect the receptors after exposure of the tissue to the drug for more than an hour

The only twitch enhancing factor which remained after PBA was K and the possibility that K is involved in the twitch response should not be overlooked This does not however preclude that electrical stimulation of the postganglionic nerves liberates NA even if it does not serve to elicit the twitch but rather the forthcoming phasic contraction (*cf* Hedqvist 1973) NA in fact inhibits the twitch presumably by prejunctional α as well as β stimulation (Hedqvist and Euler 1974)

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References

AMBACHE N and M A ZAR Evidence against adrenergic motor transmission in the guinea pig vas deferens *J Physiol* (Lond) 1971 *216* 359—389
EULER U S v and P HEDQVIST Effects of Substance P on the response of guinea pig vas deferens to transmural nerve stimulation *Acta physiol scand* 1974 *90* 651—653
FURNESS J B and T IWAYAMA The arrangement and identification of axons innervating the vas deferens of the guinea pig *J Anat* 1972 *113* 179—196
HEDQVIST P Role of the α receptor in the control of noradrenaline release from sympathetic nerves *Acta physiol scand* 1974 *90* 158—165
HEDQVIST P and U S v EULER To be published 1974
SJOSTRAND N O and G SWEDIN Potentiation by smooth muscle stimulants of the hypogastric nerve vas deferens preparation from normal and castrated guinea pigs *Acta physiol scand* 1968 *74* 472—479
TREGEAR G W H D NIALL J T POTTS JR S E LEEMAN and M M CHANG Synthesis of Substance P *Nature New Biol* 1971 *232* 87—89

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From the Departments of Anatomy* and Pharmacology ** University of Helsinki and the Institute of Biomedical Science *** University of Tampere Finland

Ultrastructure of Synaptosomes from Midterm Human Fetal Brain

By

LASSE KANERVA ANTTI HERVONEN and ANJA H TISSARI

Isolated nerve endings (synaptosomes) prepared from brain homogenates have been used in morphological biochemical and pharmacological studies to clarify different aspects of brain function (Whittaker 1965 De Robertis 1966, Tissari and Bogdanski 1971) The use of subcellular fractionation techniques has also proved of value in studies of the brain development of the rat the pig and the chick (for ref see Tissari 1973 1974 Hervonen *et al* 1974) Recently a paper appeared dealing with the ultrastructure of synaptic complexes from postnatal humans (Wannamaker *et al* 1973) but to our knowledge the fine structure of synaptosomes from human fetuses has not previously been described This is a report on the fine structure of the C layer (main synaptosome layer) of the crude mitochondrial fraction (De Robertis *et al* 1962) isolated from the frontal and occiptal hemisphere of the midterm human fetus

Midterm human fetuses (age 15—16 weeks) were obtained from legal interruptions of pregnancy performed by laparotomy The brains were immediately removed and subfractions were prepared from the frontal and occipital hemispheres as previously described (see Hervonen *et al* 1974) The crude mitochondrial fraction was re uspended in 0 32 M sucrose and layered over a discontinuous sucrose gradient consisting of layers of 1 4 1 2 1 0 and 0 8 M sucrose The gradient was centrifuged at 55 000 × g for 120 min in a Spinco Model L ultracentrifuge equipped with a SW 25 1 rotor The fractions of sucrose gradient were removed by aspiration and diluted with enough water to sucrose concentration 0 32 M for subfractions A and B and 0 55 M for C D and E After standing for 20 min the suspensions were centrifuged for 30 min at 15 000 × g except subfractions A and B centrifuged at 40 000 × g Pellets were resuspended in 14 ml of Krebs bicarbonate solution All procedures were carried out at 0 —4 Samples were fixed as suspensions in 3 % $KMnO_4$ (final concentration) (Richardson 1966 Hokfelt 1971) in ice-cold 0 1 M phosphate buffer (pH 7 0) After 30 to 60 min the fractions were centrifuged at 10 000 × g for 15 min The resulting pellets were washed in 0 1 M phosphate buffer for 1 to 12 h dehydrated and embedded in Epon Araldite The sections were viewed and photographed unstained with a Philips EM 300 operated at 40 kV In 1 expt the C fraction was incubated with 5 OHDA After prewarming the incubation mixture for 8 min 5 OHDA in final concentration of 10 µg/ml was added and incubation was performed at 37 C for 15 min using a metabolic shaker

The 5 layers obtained after gradient centrifugation of crude mitochondrial fraction became equilibrated at the same densities reported originally by De Robertis *et al* (1962) in adult rats In the present study the preliminary results of the main



Fig 1 C layer from the frontal hemisphere of a 16 week old human fetus Most of the processes (P) are devoid of synaptic vesicles but three endings (S) show a moderate number of small and some large (arrow) agranular vesicles M mitochondrion 5 OHDA incubation Photomontage ×26 700

Fig 2 C layer from the occipital hemisphere of the same fetus A mature looking synaptosome containing numerous small and some large (arrow) agranular vesicles 5 OHDA incubation ×42 000

synaptosome fraction the C layer are reported Fraction C (Fig 1 2) showed mature synaptosomes immature synaptosomes processes without synaptic vesicles and unidentified membranous debris The number of synaptic vesicles varied greatly in the processes Most of the processes were devoid of synaptic vesicles In the immature synaptosomes the number of vesicles was small and the mature synaptosomes were filled with small agranular vesicles (Fig 2) A few large agranular vesicles were noticed among the small synaptic vesicles (Fig 1 2) but small or large dense core vesicles were not observed not even after 5 OHDA (10 μg/ml) incubation The number of processes containing synaptic vesicles was smaller than in the brain stem and hemispheres of the 1 day old rat which showed synaptic vesicles in 10 to 30 % of the processes (Hervonen *et al* 1974 to be publ)

Synapses in the human fetus have been described in the adrenal medulla the superior cervical ganglion and the carotid body at the age of 11 to 13 weeks (for references see Kanerva *et al* 1974) and in the central nervous system as early as at the age of 8 5 weeks (Molliver *et al* 1973) of gestation Using fluorescence microscopy monoamine neurons were found at the age of 7 weeks in the human fetal brain (Olson *et al* 1973) and varicose fibers suggestive of axon terminals at the age

of 10 weeks Thus it is evident that morphologically *mature looking synapses can* be found early in development in the human fetus

It was calculated by Olson *et al* (1973) that regarding the monoamine neurons the human fetuses at the age of 7 to 16 weeks correspond to that of the prenatal rat (days 15 to 21) while the human fetuses at the age of 17 to 23 weeks corresponded to the newborn rat This is in agreement with the finding that synaptosomes in the 1 day old rat (Hervonen *et al* 1974) are more mature than those of the midterm (15—16 weeks) human fetus

Only limited possibilities exist at the moment to differentiate between different transmitter substances in nerve endings by means of electron microscopy It has been postulated that the small granular vesicles (diameter 500 Å) are characteristic of monoaminergic nerve endings (Hokfelt 1971) while the cholinergic endings have agranular vesicles (Whittaker 1965) Flattened vesicles after aldehyde fixation might be characteristic of at least some synaptic bulbs of inhibitory function (see *e g* Gray 1969) In the present study only one type of nerve ending has been noticed so far and synaptosomes with the characteristic of monoamine neurons were not observed although 5 OHDA incubation was used One reason might be the immaturity of the granual pool of the nerve endings

References

DE ROBERTIS E Adrenergic endings and vesicles isolated from brain *Pharmacol Rev* 1966 *18* 413—424

DE ROBERTIS E A PELLEGRINO DE IRALDI G R DE LORES ARNAIZ and I SALGANICOFF Cholinergic and non cholinergic nerve endings *J Neurochem* 1962 9 23—35

GRAY E G Electron microscopy of excitatory and inhibitory synapses A brief review Progr *Brain Res* 1969 *31* 141—155

HERVONEN A L KANERVA A H TISSARI and B V A SUURHASKO Ultrastructure of synaptosomes from one-day old and adult rat brain stem *Cell Tiss Res* 1974 *148* 535—550

HOKFELT T Ultrastructural localization of intraneuronal monoamines Some aspects on methodology *Progr Brain Res 1971 34* 213—227

KANERVA L A HERVONEN and H HERVONEN Morphological characteristics of the ontogenesis of the mammalian peripheral adrenergic nervous system with special remark on the human fetus *Med Biol* 1974 *52* 144—153

MOLLIVER M E I KOSTOVIC and H VAN DER LOO The development of synapses in cerebral cortex of the human fetus *Brain Res* 1973 *50* 403—407

OLSON L L O BOREUS and A SEIGER *Histochemical demonstration and mapping of* 5 hydroxytryptamine and catecholamine containing neurons systems in the human *fetal* brain *Z Anat Entwickl Gesch* 1973 *139* 259—282

RICHARDSON K C Electron microscopic identification of autonomic nerve endings *Nature (Lond)* 1966 *210* 756

TISSARI A H Serotoninergic mechanisms in ontogenesis In *Fetal Pharmacology* 237—257 Ed L Boreus Raven Press New York 1973

TISSARI A H Pharmacological and electronmicroscopic maturation of serotoninergic synapses in ontogenesis *Med Biol* 1974 (In press)

TISSARI A H and D F BOGDANSKI Effects of inorganic *electrolytes on the membrane* transport and metabolism of serotonin and norepinephrine by synaptosomes *Prog Brain Res* 1971 *34* 291—302

WANNAMAKER B B S E KORNGUTH C SCOTT A W DUDLEY A KELLY *Isolation and ultrastructure of human synaptic complexes J Neurobiol* 1973 *4* 513—555

WHITTAKER V P The application of subcellular fractionation techniques *to the study of* brain function *Progr Biophys molec Biol* 1965 *15* 39—96

Acta physiol scand 1974 92 289—302

From the King Gustaf V th Research Institute and Department of Internal Medicine
Huddinge Hospital Sweden

Synthesis of Serum Albumin in Isolated Rat Liver Perfused by a Synthetic Medium with Fluorocarbon FC—80 Emulsion as Oxygen Carrier

By

VERA NOVAKOVA G BIRKE and L O PLANTIN

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Abstract

NOVAKOVA V G BIRKE and L O PLANTIN *Synthesis of serum albumin in isolated rat liver perfused by a synthetic medium with fluorocarbon FC-80 emulsion as oxygen carrier* Acta physiol scand 1974 92 289—302

A fluorocarbon emulsion FC 80 has been used as a substitute for erythrocytes in a synthetic nutrition medium in *in vitro* perfusions of isolated rat livers The rate of albumin synthesis was estimated After the first hour albumin was synthesised at a constant rate of 0 52±0 17 mg/h per 100 g of the body weight of the liver donor ^{14}C lysine was incorporated into the circulating proteins linearly after the first hour Other parameters tested were levels of circulating proteins urea nitrogen glucose glutamic pyruvic transaminase excretion of bile weight changes of the liver and level of glycogen in liver tissue after perfusion Histological examinations were also performed The function of the liver was comparable with the findings of other investigators who used whole blood as the perfusate As far as we are aware this is the first time that a rat liver has been maintained in good functional condition for over 6 hours during in vitro perfusions with a synthetic medium that included an erythrocyte substitute The method seems to offer great advantages for the study of metabolic processes
Key words Fluorocarbon Perfusion Isolated liver Liver

The replacement of blood by a simpler chemically defined more standardised and less expensive fluid has become desirable in different fields such as perfusion studies preservation of organs for transplantation or as emergency blood

One of the main difficulties was to obtain an adequate substitute for erythrocytes Clark and Gollan (1966) found that a fluorocarbon liquid produced by the 3M Company has properties that makes it suitable for this purpose Fluorocarbons (FC) are nonpolar inert liquids with an oxygen-carrying capacity exceeding that of blood Geyer *et al* (1968) replaced red cells with emulsified fluorocarbons and reported that rats survived for 8 h The fluorocarbon has been used successfully as oxygen carrier in perfusions of rat brain (Sloviter and Kamimoto 1967) and other organs (Beisang *et al* 1970 Geyer 1970 Lowenstein *et al* 1970 Brown and Hardison

(1972) and Triner *et al* (1970) used fluorocarbon for perfusion of rat liver

In the present investigation a new type of synthetic medium was used for continuously perfusing the isolated liver of the rat The medium contained FC-80 fluorocarbon as a substitute for erythrocytes and Difco TC 199 medium as a substitute for plasma

Immunological methods and isotope techniques were used to study the relative rate of albumin synthesis in this perfusion system Several criteria were established to assess the physiological condition of the perfused organ Possible changes in the function of the liver were followed by measurement of the concentration of the urea nitrogen glucose and total proteins in the perfusate as well as the glycogen content of the liver Histological studies were made in parallel

Experimental

Materials The chemicals used were Difco TC-199 medium obtained from Difco Laboratories Michigan USA This medium is the dried formulation of the chemically defined medium of *Morgan et al (1950) Details are available from the manufacturer FC 80 fluorocarbon* was obtained from the 3M Company chem div Minneapolis Minnesota USA L-α Lecithin from egg yolk commercial grade type II E Puromycin diHCl kits for determination of glutamic pyruvic transaminase (GPT) by the Sigma Frankel method (Sigma technical bulletin No 505) kits for determination of glucose (Sigma technical bulletin No 510 Sigma Company USA) Kits for determination of urea nitrogen (Hyland's UN test) were obtained from AB Kemila Preparat Stockholm Sweden Serum albumin human 20 solution for injections was from KABI Sweden Heparin solution 5000 IU/ml was used (Vitrum Sweden ^{14}C L-Lysine monochloride (specific radioactivity 312 mCi/mmol was obtained from the Radiochemical Centre Amersham UK ^{131}I human albumin was made by tagging purified human albumin with ^{131}I according to McFarlande 1958) The specific antiserum against rat albumin was delivered by Nordic Pharmaceutical and Diagnostics the Netherlands Mebumalnatrium was from ACO Company Stockholm Sweden

Media Two types of media were used One for pre perfusion and the other for perfusion of the organ The pre perfusion medium consisted of 1 1 g of TC 199 Difco *medium* and 500 IU *Heparin in 100 ml of double-distilled water The perfusion medium was made up from* 1 1 g of TC 199 Difco medium 1 5 g of lecithin from egg yolk 10 ml of emulsified fluorocarbon FC-80 4 ml of (20) human albumin and 100 mg of dextrose in 100 *ml* of double-distilled water final volume

Preparation of the medium 1 5 g of lecithin in 30 ml of double distilled water were kept overnight at 4 C Hydrated lecithin was emulsified by an ultrasonic generator Biosonic III) at full power 300 W at 20 KHz *in* two 15-second exposures with cooling *in an ice* bath between the exposures FC 80 fluorocarbon was slowly added to the lecithin emulsion and the whole system was then agitated by the ultrasonic generator Excessive heating of the emulsion must be avoided otherwise the solution will coagulate and form small aggregates Sonication was performed for 15 the emulsion cooled for 3 min and the whole procedure repeated ten times This method produced a suitable and relatively stable emulsion After sonication the mixture was filtered through a 3 nm Millipore filter and mixed with the dissolved TC 199 medium Human albumin and 100 mg of dextrose were added The emulsion was gassed with O_2 CO_2 95 5 v/v for 30 min The pH was adjusted to 7 4 with sterilized 1 M $NaHCO_3$ Finally the mixture was *made up to 100 ml with double-distilled* water ^{14}C L lysine monochloride 1 25 μCi was added to 45 ml of the medium

Animals Male Sprague Dawley rats weighing 350—500 g were maintained on commercial laboratory chow pellets Access to food and water was *ad libitum* The rats were anesthetized with Mebumal sodium which was injected intraperitoneally 0 30 mg per kg b wt) Then after 500 IU of Heparin in 1 ml of 0 9 NaCl were injected into the femoral vein

Operative procedure and apparatus This was described by Miller *et al* (1951) but has been slightly modified The bile duct and portal vein were cannulated and the latter was thoroughly rinsed out *in situ* with about 20 ml of oxygenated pre perfusion medium NaCl

The liver was rapidly dissected transferred to a Petri dish containing saline (0 9

sterile 37 C) and rinsed out at a rate of 30—40 ml/min) with 100—150 ml of oxygenated pre perfusion medium until all the blood was washed out The isolated liver was rapidly weighed and transferred to the thermostated chamber comprising part of the perfusion apparatus in which the medium had already been circulating for 1 h

The perfusion aparatus (Fig 7) consists of an organ chamber an oxygenator a graduated container for the perfusion medium a water condenser and a system of polyethylene tubings with filter The oxygenated medium was pumped continuously through the liver by a peristaltic pump An ordinary reflux condenser was used for oxygenation of the perfusate by a humidified mixture of O_2 CO (95 5 v/v) The upper part of the oxygenator was connected to a water cooled glass condenser Changes in the actual volume of the perfusate (needed for the calculation of the results) were observed on the graduated medium container The flow rate (24 ml/min) and temperature (37 C) were kept constant Changes in the fluid pressure of the portal vein could be observed Initially it was between 2—5 cm of H O

Analysis of perfusate The perfusion was performed for 6 h and samples of the medium were collected each hour They were centrifuged at about 2500 g for 40 min and the supernatant was used for further assays GPT was measured by the Sigma Frankel method Circulating proteins were determined by the method of Lowry *et al* (1951) The glucose level was determined by the enzymatic colorimetric method published by Sigma Urea nitrogen was measured by Hylands UN test Rat albumin concentration was determined by the immunological method of Mancini *et al* (1965)

Rat albumin was isolated by a combination of zone electrophoresis on polyvinyl chloride and separation on Sephadex G 100 and used as a standard

Using the Ouchterlony (1953) double-diffusion technique we obtained single continuous precipitin lines of antiserum against purified rat albumin and rat serum No precipitation was detected against human serum albumin

Exact determination of the initial wet weight of the liver was difficult because of the pre perfusion of the organ *in situ* Consequently the albumin synthesis has been related to the b wt. of the donor animal and is expressed in mg/h/100 g of b wt

Column chromatography on Sephadex G 25 was used to separate proteins from amino acids The supernatant (0 4 ml) was added to the column (10×200 mm) The column was previously calibrated with a solution containing a known amount of albumin and amino acids After elution with redistilled water 1 ml fractions were collected

^{14}C lysine radioactivity incorporated into the proteins was counted in a Packard Liquid scintillation spectrometer Nine ml of Instagel (Packard Instrument) were added to each 1 ml fraction Quench corrections were made by adding a known amount of ^{14}C activity

Analysis of the liver After 6 h of perfusion the liver was removed weighed and samples of the tissue were taken for histological and chemical studies The glycogen content of the liver was determined by the anthron method (Hassid and Abraham 1957)

Control experiments In 1 expt puromycin dihydrochloride (15 mg/95 ml) was included in the perfusion fluid from the beginning and in addition 10 mg were infused between 30 min and 4 hr of the perfusion time in accordance with John and Miller (1966) This was done to observe the comparative effects of a known inhibitor of protein synthesis

The possible catabolism of I labelled human serum albumin was studied in one perfusion 10 μC of a preparation labelled with 1 atom I per albumin molecule was added to the medium and the perfusion performed Each hour during 7 5 h samples of medium were collected centrifuged and proteins and amino acids separated as described above I radioactivity in proteins and amino acids of the perfusate and in the samples of liver tissue (taken at the end of the perfusion) was measured in an ordinary scintillation counter

To show the importance of fluorocarbon in making a successful perfusion and for the activity of the liver fluorocarbon was not added to the perfusion medium in one experiment

Results

Eleven experiments were made in which the liver was perfused for 6 hours The condition of the liver was defined and unsuccessful experiments were easily distinguished by means of the measured parameters Average values for 8 normal perfusions are presented and compared with those of 3 unsuccessful and of 3 control perfusions

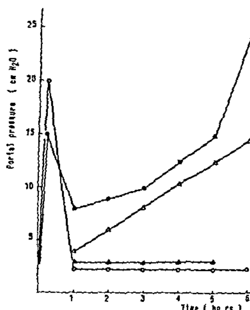


Fig 1

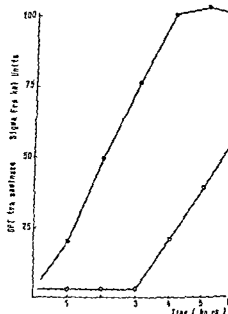


Fig 2

Fig 1 The pressure in the portal vein (in terms of cm H_2O) during the perfusion of the normal liver is indicated by O the unsuccessful perfusion by ● perfusion of the puromycin treated liver by ▲ and perfusion without fluorocarbon by △ In unsuccessful perfusions the liver damage was due to the mechanical injury of the liver lobe during the operation

Fig 2 The GPT level in the medium expressed in terms of Sigma Frankel units O normal experiment ● unsuccessful experiment (liver damage was due to the mechanical injury of the liver lobe during the operation)

General properties of the system Experiments were defined as normal when the portal pressure was stable and the GPT level increased slowly and continuously during the entire perfusion Furthermore the liver was in a good condition as judged by visual observation at the end of the perfusion and a minimal increase in the liver weight (oedema) was observed (0—10 % of the initial wet weight of the liver) A pink colour wet surface absence of discoloured patches and production of bile were other signs of a good condition of the liver

Unsuccessful experiments were characterized by increased portal pressure followed by a sudden high increase in the GPT level The liver was enlarged with discoloured patches and bile was not produced The wet weight of the liver increased by more than 40 % over the initial weight Control perfusions were also made as described above No rat albumin was produced when puromycin was added or when the fluorocarbon was omitted from the perfusion medium

When ^{131}I labelled human albumin was added to the perfusion medium no radioactivity was found in the amino acid of the perfusate 3.5 % of the radioactivity was found in the liver tissue

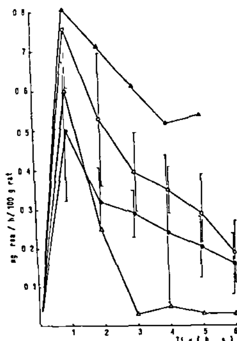


Fig 3

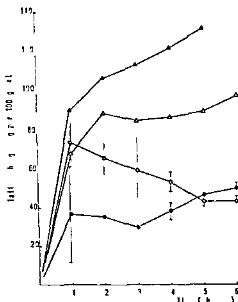


Fig 4

Fig 3 The changes in the urea nitrogen content expressed as mg per h of perfusion per 100 g of liver donor b wt O normal experiments (8 perfusions) ● unsuccessful experiments (3 perfusions) ▲ puromycin was added (1 perfusion) △ fluorocarbon was not added (1 perfusion) Mean values ± standard deviations are shown

Fig 4 The cumulative changes of glucose in the perfusion medium expressed as mg per 100 g of liver donor b wt O normal experiments (8 perfusions) ● unsuccessful experiments (3 perfusions) ▲ experiment with puromycin treated liver (1 perfusion) △ fluorocarbon was not added to the medium (1 perfusion) Mean values ± standard deviations are shown

The criteria of general condition and viability

Portal pressure Fig 1 shows the fluid pressure in the portal vein during the normal the unsuccessful the puromycin treated perfusion and the perfusion without fluorocarbon In a normal experiment the pressure was higher after the perfusion was started but decreased within 10 min and then remained constant In an unsuccessful perfusion (the liver damage was due to mechanical injury of the liver lobe during the operation) the portal pressure increased Even minute injuries caused an immediate increase in the portal pressure In the puromycin treated liver experiment the portal pressure was low and stable after the initial period of perfusion In the perfusion without fluorocarbon the portal pressure increased gradually and continuously during the entire perfusion

GPT activity This was expressed in terms of Sigma Frankel units Fig 2 shows the results of one normal and one unsuccessful perfusion In the normal perfus

TABLE 1 *Rates of rat albumin synthesis* Comparison of rates of synthesis of rat serum albumin determined for *in vitro* perfusions and *in vivo* by different authors Different types of perfusion media were used in the perfusion studies

In vitro perfusions			Rates of albumin synthesis		
Authors	Rats	Perfusion medium	mg/h per whole rat	mg/h per 100 g rat	mg/h per 1 g liver
Mattern *et al* (1972)	Starved	*Eagle s medium*	*mean ± S D*	*mean ± S D*	*mean ± SD*
		without erythrocytes	0 98	0 66	0 18
		with erythrocytes	1 27 0 40	0 78 0 37	0 23 0 01
	Fed	without erythrocytes	1 36	0 76	0 17
		with erythroctyes	2 76 0 37	1 65 0 33	0 39 0 06
Hoffenberg *et al* (1971)	Fed	Rabbit blood	mean (ranges)	mean (ranges)	mean (ranges)
	normal diet	(plasma + erythroctyes)	4 0 (2 6 – 4 6)	1 1 (0 8 – 1 3)	0 3
	nonprotein *diet*	(plasma + erythrocytes)	1 4 (0 9 – 1 7)	0 9 (0 6 – 1 1)	
John and Miller (1966)		Rabbit blood			0 5
Katz *et al* (1968)				10 0	2 4
			mean ± S D	mean ± S D	mean ± S D
This investigation	Fed normal diet	Synthetic with FC 80	1 87 0 39	0 59 0 17	
		Synthetic without *FC-80*	*0*	*0*	
		Synthetic with FC-80			
		and added puromycin	*0*	*0*	
		and aded puromycin	*0*	*0*	
Screiber *et al* (1971)		*In vivo* calculated			0 8

the activity was low for the first 3 h and increased gradually and continuously during the following 3 h In the unsuccessful perfusion (the liver was mechanically injured during the operation) the injury was indicated by a sudden increase in the GPT level The results of the perfusion with added puromycin were similar to those of the normal experiments In the perfusion without fluorocarbon the GPT levels were similar to those of the unsuccessful experiments

Changes in the wet weight of the liver The liver was weighed both before the start and after the completion of the perfusion The increase in weight was expressed as a percentage of the initial weight before the perfusion The changes in weight were between 0 and 10 % in normal experiments This was increased by 40 % in unsuccessful experiments and by 9 % in the perfusion with puromycin and by 55 % in the perfusion without fluorocarbon

Basic characteristics of the liver

The secretion of bile In normal perfusions the bile was secreted in a decreasing rate until between the 5th and the 6th hour of perfusion Totally between 0 7—1 4 ml of bile was secreted during 6 h In the unsuccessful experiments the bile production ceased immediately after the injury was observed

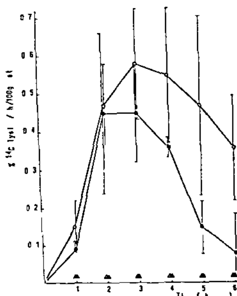


Fig 5

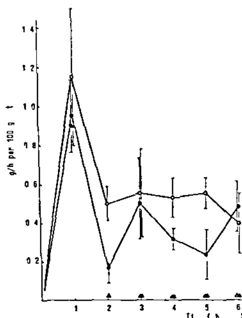


Fig 6

Fig 5 Incorporation of ^{14}C lysine into the circulating proteins expressed as net changes (in terms of the percentage of the initial amount of added radioactivity) per h of perfusion per 100 g of liver donor b wt O normal experiments (8 perfusions) ● unsuccessful experiments (3 perfusions) ▲ puromycin was added (1 perfusion) △ fluorocarbon was not added 1 perfusion) Mean values ± standard deviations are shown

Fig 6 Synthesis of rat serum albumin expressed as changes in albumin content in terms of mg per h of perfusion per 100 g of liver donor b wt O normal experiments (8 perfusions) unsuccessful experiments (3 perfusions) ▲ puromycin was added (1 perfusion) △ fluorocarbon was not added (1 perfusion) Mean values ± standard deviations are shown

Total proteins in perfusate An initial level of human albumin of 0 8 (w/v) % was necessary to maintain a sufficient colloid osmotic pressure and a stable emulsion of the fluorocarbon Consequently it was difficult to measure small changes in the concentration of the total proteins However the results of 8 normal experiments showed that the level of the circulating proteins decreased during the first and the second hour of perfusion and then remained at the same level for the duration of the experiment In the unsuccessful experiments there was invariably a rapid significant increase in the protein concentration of the perfusion fluid just after an injury was observed

Urea nitrogen The results are expressed as changes in mg/h of perfusion per 100 g of liver donor body weight (Fig 3) Urea was synthesized at a decreasing rate during the experimental period in the 8 normal perfusions at a decreasing but lower level in 3 unsuccessful perfusions and decreasing but at a higher level in the perfusion with puromycin In the experiment without fluorocarbon the rate of synthesis decreased until the 2nd hour and thereafter synthesis ceased

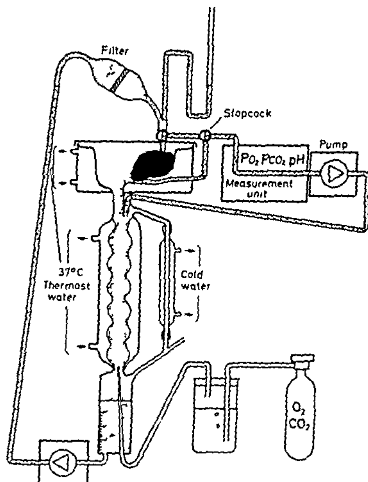


Fig 7 Schematic diagram of the perfusion apparatus The unit for measurement of pH PO_2 PCO_2 was recently added to the apparatus

Glucose The cumulative changes in glucose in the perfusate expressed as mg per 100 g of liver donor body weight are shown in Fig 4 During the first hour of perfusion a similar high increase in the glucose concentration occurred in all types of perfusion In the unsuccessful experiments the increase was lower (not significantly) than that in normal experiments In the circulating medium the initial concentration of glucose was 0 2 % (w/v) Owing to the high initial level of glucose present after the first hour of perfusion small changes in concentration were difficult to measure After the first hour however for a group of normal perfusions the results show a decreasing tendency for changes in concentration whereas in unsuccessful and control perfusions concentration was either constant or tended to increase slightly

Glycogen in the liver tissue The results are expressed as mg of glycogen per 1 g of wet weight of the liver The 3 nonperfused livers contained 50 mg of glucose per g of liver whereas after 6 hours of perfusion the content was reduced by 80 % to 10 mg/g of liver in the group of normal experiments No measurable amount of glycogen was found in the unsuccessful and the control experiments

Histology Normal experiments The histological picture shows only a partial vacuolization or hyalin degeneration Serious injury necroses and degeneration of cells were found in the unsuccessful experiments

Synthesis of serum protein and albumin

a Incorporation of ^{14}C *lysine radioactivity into circulating proteins* The results expressed as changes (in the percentage of the initial amount of added radio activity) per hour of perfusion per 100 g of liver donor body weight are shown in Fig 5 During the first hour 0 15 ± 0 08 % of the radioactivity was incorporated into the protein fraction in normal and unsuccessful perfusions

In normal experiments the incorporation varied slightly (but not significantly) and was 0 52 ± 0 18 % per hour per 100 g of b wt after the first hour of perfusion During the last hour the incorporation decreased slightly (but not significantly) to 0 38 ± 0 14 % g of b wt The rate of incorporation in the unsuccessful experiments decreased from the third hour of perfusion The incorporation was completely inhibited in the perfusion with puromycin There was no incorporation in the perfusion without fluorocarbon

b Synthesis of rat serum albumin Changes in content of rat serum albumin (expressed as mg per hour of perfusion per 100 g of liver donor b wt are shown in Fig 6 During the first hour of perfusion there was a rapid increase of albumin content (1 1 ± 0 3 mg/h per 100 g of b wt) in the 8 normal and 3 unsuccessful expts and in those where puromycin was added In the perfusion without fluoro carbon the increase was lower (0 39 mg/h per 100 g of b wt)

In 8 normal expts the albumin was synthesized continuously at a constant rate (0 52 ± 0 17 mg/h per 100 g of b wt or 1 9 ± 0 6 mg/h per whole animal) after the first hour of perfusion During the 6th hour the rate of synthesis was slightly (but not significantly) decreased In the three unsuccessful experiments the rate of synthesis between the second and the sixth hour varied In the perfusion without fluorocarbon and in the experiment where puromycin was added the additional albumin synthesis was not measurable after 2 h of perfusion

Discussion

The preliminary experiments were carried out by Dolezalova (unpublished studies) She reported that the survival of the isolated perfused liver was between 6 and 12 h at 37° C At lower temperatures (24° 26° C) survival was correspondingly longer At 24° C it was possible to keep the liver viable for 41 h

The assessment of the condition and degree of physiological normality of a perfused organ can be based on observations and measurements of a number of parameters The choice of representative parameters and the method of their evaluation are important when the condition of the liver and the value of the perfusion medium as a blood subsitute are to be assessed

Three types of parameters were used

1 Criteria of general condition and viability Parameters which can give us acceptable information for routine use on the general condition and viability of the liver They must enable differentiation between a normal and a damaged liver
2 Basic characteristics of the liver The parameters which should provide information on several basic functions of the liver
3 Synthesis of circulating proteins and albumin One important metabolic function (such as albumin synthesis) which is dependent on the cooperation of several complicated enzyme systems should be studied in greater detail

These types of parameters are discussed and compared with the results of other perfusion systems and for whole animals

1 Criteria of general condition and viability

When the liver is damaged (for example by anoxia) among the first measurable changes are the leakage of some intracellular enzymes the water uptake and the altered vasoconstriction We therefore measured the amount of GPT the changes in the wet weight of the liver and the changes in the fluid pressure in the portal vein A rise of portal pressure indicate either a block in the circuit or increased hepatic vascular resistance This was a sensitive sign of the condition of the liver which agrees with the findings of Cohen *et al* (1973)

GPT A sudden increase in the GPT level was a sensitive indication of liver cell injury GPT was always higher during the last hour of perfusion indicating some damage of the hepatic cells Similar results were obtained by Kvetina and Guaitani (1969) already after 60 min of perfusion with blood

The water uptake The wet weight of the healthy liver was the same or slightly (not significantly) increased at the end of the perfusion It means that either the composition of the liver was unchanged or the changes were such that the total weight was unchanged There are considerable difficulties in estimating all the factors which account for the changes in weight Water uptake is certainly the most important factor but the release of liver components as for instance glycogen is also important

2 Basic characteristics of the liver

The secretion of bile The bile flow during the perfusion is comparable with that reported in the studies of Miller *et al* (1951) Brauer *et al* (1951) Fisher and Kerly (1964) and Kvetina and Guaitani (1969) and was less than observed *in vivo* by Brauer *et al* (1951)

Urea formation indicates a normal liver function The reason for the decreasing rate of formation is not known at this stage

The glucose and the glycogen An increase of glucose in the perfusion fluid during the first hour of perfusion and a considerable reduction of the liver glycogen was observed It represents the most significant change Similar findings were observed by Brauer *et al* (1951) Burston and Apsey (1967) Penthos *et al* (1966 a b) and Kvetina and Guaitani (1969) Bloxam (1971) found that 95 % of the liver glycogen had been lost already during the operation and transfer of the liver Fisher and Kerly (1964) did not observe any change in liver glycogen

The possible explanation of massive glycogenolysis which obviously occurs during the transfer of the liver to the perfusion apparatus and the initial period of the perfusion can be a glycogenolytic action of catecholamines released during this period The response to stress of all kinds is as is well known characterized by activation of the sympathetic nervous system which results in excessive secretion of catecholamines

Bromosulfophtalein clearance (BSP) The disappearance of the BSP which is usually considered as a good indicator of liver function was studied in preliminary experiments It was rapid (Dolezalova unpublished investigation) and in accordance with the studies of Brauer *et al* (1951) Gorman *et al* (1967) Bar (1962) and Kvetina and Guaitani (1969)

3 Synthesis of circulating proteins and albumin

a *Incorporation of ^{14}C lysine to circulating proteins* This indicated continuous and relatively constant protein synthesis after the first hour of perfusion (The lower incorporation during the first hour can be explained by the release of presynthesized proteins from the liver tissue to the perfusion medium) The total amount of radioactivity incorporated varied between 5 and 8 % which results agree with those of Miller *et al* (1951) who found that about 2—8 % of the total radioactivity was incorporated into the circulating proteins during six hours of perfusion with whole blood

b *Synthesis of rat serum albumin* Measurement of the net changes of rat serum albumin per hour indicated a continuous and constant albumin synthesis after the first hour of perfusion The rapid high increase af albumin concentration during the first hour of perfusion was observed also by John and Miller (1966) and by Mattern *et al* (1972) A comparison of the synthesis rate for albumin in the rat found by others *in vitro* and *in vivo* is presented in Table 1 The synthesis rate obtained in our experiments is evidently comparable to but lower than that found by other authors using erythrocytes or whole blood The reason why we do not obtain a higher albumin production with oxygen carrier than Mattern *et al* (1972) without any could be that there is some other limiting factor for the synthesis

It may be that Eagle s medium is superior to the Difco TC 199 medium we have used or that our oxygenator is inefficent Some recent measurements point to the latter as we find an extraordinarily low oxygen concentration in the medium after

The assessment of the condition and degree of physiological normality of a perfused organ can be based on observations and measurements of a number of parameters The choice of representative parameters and the method of their evaluation are important when the condition of the liver and the value of the perfusion medium as a blood subsitute are to be assessed

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problems still remain to be solved and that studies of additional metabolic para meters and functions of the liver and of other isolated organs should be made

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References

BAR N Theses la faculte de sciences del universite Lille 1961

BEISANG A. A J L. FEEMSTER, R. H DIETZMAN H LCHIDA J E. CARTER E F GRAHAM and R G LILLEHEI Damage assay of kidneys frozen by intraarterial perfusion with a fluorocarbon *Fed Proc* 1970 *29* 1782—1788

BLOXAM D L. Nutritional aspects of amino acid metabolism. *Brit J Nutr* 1971 *26* 393—422

BRAUER, R. W R L. PESSOTI and P PIZZOLATO Isolated rat liver preparations bile production and other basic properties *Proc Soc exp Biol* (NY) 1951 *78* 174—181

BROWN H and W G M HARDISON Fluorocarbon sonicated as a substitute for erythrocytes in rat *Surgery* 1972 *71* 388—394

BURSTON D and M E. APLEY Glycoprotein synthesis by the perfused livers from normal and tumour bearing rat *Brit J Cancer* 1967 *21* 801—810

CLARK L. C and F GOLLAN Survival of mammal breathing organic liquids equilibrated with oxygen at atmosphere pressure *Science* 1966 *152* 1755—1756

COHEN R D R A ILES and H M LLOOY *Isolated organ perfusion* H D Ritchie and J D Hardcastle eds 1973 131—131

FAUVERT R E. J WEBER and J P BENHAMOU Bromsulphalein fractional clearance *Biochem Clin* 1964 *3* 39—45

FISHER M M and M KERLY Amino acid metabolism in the perfused rat liver *J Physiol* (Lond) 1964 *174* 272—294

GEYER R. P Whole animal perfusion with fluorocarbon dispersions *Fed Proc* 1970 *29* 1758—1763

GEYER, R. P R G MONROE and K TAYLOR, Survival of rats having red cells totally replaced with emulsified fluorocarbon *Fed Proc* 1968 *27* 384—384

GORMAN C A E N FLOCK and C A OWENS Penicillin induced changes in the function of the isolated perfused rat liver Alterations in the thyroxine metabolism and sulfobromophtalein excretion *Endocrinology* 1967 *80* 247—254

HASSID W Z and S ABRAHAM *Methods in Enzymology* S P Colowick and N O Kaplan eds Academic Press Inc New York 1957 III 34—54

HOFFENBERG R. A H GORDON and E G BLACK Albumin synthesis by perfused rat liver *Biochem J* 1971 *122* 129—134

JOHN D W and L L MILLER, Influence of Actinomycin D and Puromycin on net synthesis of plasma albumin and fibrinogen by the isolated perfused rat liver *J biol Chem* 1966 *241* 481—4874

KATZ J A L SELLERS and G BONORRIS *Stoffwechsel der isolierten perfundierten Leber* (W Staib and R Scholz eds) Berlin—Heidelberg—New York Springer 1968 100—108

KVETINA J and A GUAITANI A versatile method for the *in vitro* perfusion of isolated organs of rats and mice with particular reference to liver *Pharmacology* 1969 *2* 65—81

LOVELETTEL E J C R LINCOLN J H MODELL W G AUSTEN and M B LAVER Preservation of excised canine lungs by ventilation with liquid fluorocarbon *Fed Proc* 1970 *29* 1775—1777

LOWRY O H N J ROSEBROUGH A. L. FARR and R. J RANDALL Protein measurement with the Folin phenol reagent. *J biol Chem* 1951 *193* 265—275

MCFARLANE A S Efficient trace labelling of proteins with iodine *Nature* Lond 1958 *182* 53—53

MANCINI G A O CARBONARA and J F HEREMANS Immunochemical quantitation of antigens by single radial immunodiffusion *Immunochemistry* 1965 *2* 235—254

MATTERN S W FROHLING and K W BOCK Albumin synthesis in isolated perfused livers from phenobarbital pretreated rat. *Naunyn-Schmiedebergs Arch Pharmacol* 1972 *273* 43—247

MILLER L. L. G G BLY M L WATSON and W F BALE The dominant role of the liver in plasma protein synthesis *J exp Med* 1951 *94* 431—453

MORGAN J F H J MORTON R C PARKER Nutrition of animal cells in tissue culture Proc Soc exp Biol (N Y) 1950 73 1—8
OUCHTERLONY O Antigen antibody reaction in gels Acta path microbiol scand 1953 32 231—240
PENTHOS J C C H WU A LEMBERG J DAUNAS M REITMAN and R LEVINE Effects of glucagon on the metabolism of lipids and on urea formation by the perfused rat liver Diabetes 1966 15 740—748
PENTHOS J C C H WU A LEMBERG J DAUNAS B BRODOFF and R LEVINE The effect of growth hormone on the metabolism of lipids and on urea formation by the perfused rat liver Metabolism 1966 15 1109—1119
SCHREIBER, G J URBAN J ZAHRINGER W REUTER and U FROSCH The secretion of serum protein and the synthesis of albumin and total protein in regenerating rat liver J biol Chem 1971 246 4531—4538
SLOVITER H A and T KAMIMOTO Erythrocyte substitute for perfusion of brain Nature (Lond) 1967 216 458—460
TRINER L M VEROSKY D V HABIF and G G NAHAS Perfusion of isolated liver with fluorocarbon emulsion Fed Proc 1970 29 1778—1781

Acta physiol scand 1974 92 303—317

From the Department of Clinical Neurophysiology University Hospital Uppsala Sweden

Single Unit Sympathetic Activity in Human Skin Nerves during Rest and Various Manoeuvres

By

ROLF G HALLIN and H ERIK TOREBJÖRK

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Abstract

HALLIN R. G and H E TOREBJÖRK *Single unit sympathetic activity in human skin nerves during rest and various manoeuvres* Acta physiol scand 1974 92 303—317

Single unit activity in sympathetic nerve fibres was studied in microelectrode recordings from intact cutaneous nerves in alert man. 4 units exhibited a "spontaneous" discharge of very low frequency (not exceeding 15 imp/min) during resting conditions whereas 4 other units were silent. Mental concentration and arousal stimuli of different types caused an increase in impulse frequency of spontaneously active units and previously silent units started firing. In reflex responses to electrical shocks, deep breathing and loud noises single units often fired repeatedly in bursts, the maximal instantaneous frequency observed being 35 imp/s. Intense reflex responses to electrical stimulation were sometimes followed by a suppression of spontaneous activity for 1—2 s. Some of the units probably had a sudomotor function since the unit activity correlated well with changes in galvanic skin resistance recorded within the innervation zone of the explored nerve fascicle. The conduction velocity measured for 6 sympathetic units ranged 1.0—0.58 m/s.

It was recently shown that bursts of postganglionic sympathetic mass activity can be recorded in intact nerves of alert man (Hagbarth and Vallbo 1968). Different types of discharge patterns were observed in the sympathetic outflow to muscle nerves (Delius *et al* 1972 a, b) as compared with cutaneous nerves (Hagbarth *et al* 1972, Delius *et al* 1972 c). In these recordings it was not possible to study the firing pattern of individual units.

The purpose of this paper is to present some criteria for identification of impulses in single sympathetic units in intact skin nerves of alert man and to study the firing patterns of these units during resting conditions and in responses to various stimuli and manoeuvres.

A preliminary report of the present results has appeared previously (Hallin and Torebjörk 1970).

Methods

Subjects The investigations were carried out on 6 males ranging in age from 25 to 35 years They had no signs or symptoms of any neurological or cardiovascular disorders The subjects were familiar with the experimental situation

Median nerve One recording 5 cm proximal to the elbow (1 unit) and one recording 2 cm proximal to the radiocarpal joint (3 units)

Peroneal nerve Three recordings at the fibular head (6 units) and one recording from the superficial peroneal branch 6 cm proximal to the lateral malleolus (1 unit)

Firing patterns were studied in 8 of these 11 units (Table I) Conduction velocities were measured for 6 units three of these are included in Table I

Material

General procedure Before starting the experiments which lasted for 3—6 h the subjects were instructed to empty the bladder and bowel During the experiments the subjects sat relaxed in a comfortable semivertical position and were told to avoid active movements of the extremities Room temperature varied from 20 to 25 C

Tungsten microelectrodes were inserted manually into nerve fascicles supplying glabrous skin of the hand or non glabrous skin on the ankle or the dorsum of the foot The innervation zones corresponded with the distribution of the insertion paraesthesias experienced by the subjects on impaling a fascicle The extensions of the receptive fields were checked by recording the neural responses evoked by touch stimuli in the skin (Vallbo and Hagbarth 1968 Hagbarth *et al* 1970) Recordings from nerve fascicles supplying muscles or mixed fascicles supplying both muscles and skin were not included

Technical details The lacquer insulated tungsten microelectrodes (diameter 0.2 mm with a tapered portion about 2 mm long and with a sharp exposed tip not exceeding 30 μm) and the recording and display system were described in previous reports (Vallbo and Hagbarth 1968 Hagbarth *et al* 1970) The signal-to-noise ratio of the C unit potentials was improved by reducing the bandwidth of the display system from 0.2—10 kHz to 0.5—2 kHz (Torebjork and Hallin 1974) and 50—75 % of the remaining noise was eliminated by an amplitude discriminator (Hagbarth *et al* 1970) The nerve signals distorted by filtering are presented without calibration in the figures Occasionally a mean voltage display of the neural activity was used (Hagbarth *et al* 1970) The time constant of the integrating circuit was 0.1 s

Respiratory movements were monitored by a strain gauge attached to a band around the thorax

ECG was recorded with surface electrodes on the chest

Changes in galvanic skin resistance were recorded by two Ag AgCl electrodes in a Wheatstone bridge arrangement One electrode was attached to the skin within the innervation zone of the impaled fascicle and the other was placed approximately 20 cm outside this zone

Pulse plethysmogram was recorded by a crystal pressure transducer (EMT 510 Elema Schonander Ltd Stockholm Sweden) This was attached to an air filled plastic cylinder applied around the distal phalanx of a finger within the innervation zone of the impaled fascicle

Stimuli and manoeuvres The influence of changes in the attentative state of the subjects on the sympathetic outflow was studied during periods of 0.5—10 min when the subjects were either at rest (1) talked (2) or were subjected to mental stress (3)

1 *Resting conditions* The subjects were instructed to relax completely with their eyes closed and to breath normally The loadspeaker sound was turned off conversation was not allowed and external stimuli were avoided as much as possible in order not to disturb the subjects

2 *Talk* The subjects were allowed to converse and to listen to the loudspeaker sound of their own neural activity Unexpected loud noises and deep breathing were avoided

3 *Mental stress* The subjects were given moderately difficult problems in mental arithmetic such as consecutive subtractions of a two-digit number from a four-digit number and were urged to do this as quickly as possible Sometimes the subjects were informed that they could expect a painful stimulus (not carried out)

Other manoeuvres known to induce massive reflex responses in sympathetic fibres in cutaneous nerves (Hallin and Torebjork 1970 Hagbarth *et al* 1972 Delius *et al* 1972) were used such as unexpected nonpainful tapping on the skin loud noises painful pin pricking or deep breathing

Painful electrical stimulation was performed with a DISA stimulator unit (Type 14 E 01) delivering square wave pulses of 0.05—0.2 ms duration and an amplitude of 100—500 V at frequencies ranging from 0.1 Hz to 0.3 Hz Small stainless steel needles with a diameter of

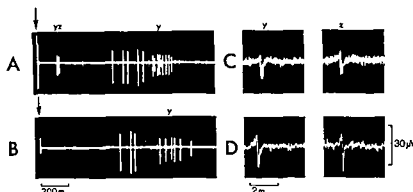


Fig 1 Sympathetic reflex discharges recorded in the median nerve at the wrist The same recording provided material also for Fig 4—7 A Response to a single painful el ctric shock in the skin within the innervation zone of the impaled fascicle (middle finger) Note repeated impulses in a large unit, x appearing at a latency exceeding 0 5 s and followed by several di charges in smaller units The latter were identical with units "y" and "z" appearing at latencies of 140 and 150 ms in the centripetally conducted C respon e as proved by collision tests (Hallin and Torebjork 1974) Conduction distance 14 cm B Reflex response to a painful stimulus in the contralateral hand C Five superimposed potentials of unit "y" and z' respectively D Single potentials of unit x Calibrations and bandwith (0 2— kHz) identical n C and D Stimulus artefacts are indicated by arrows in Fig 1 5 and 6

0 3 mm were inserted intradermally at an interelectrode distance of 4—8 mm nd ... stimulating electrodes in the skin

Nerve blocking was achieved by injection of 1 % Lidocaine close to the nerve

Results

Identification of sympathetic single unit activity in cutaneous nerves ... potentials were identified by amplitude and shape on successive d... appeared all or none in the neurogram Only units with relatively l... the order of 20—60 μV which could be easily discriminated from t... noise in the recording (about 10 μV in amplitude) and from t... sympathetic fibres were included in the study Examples of such ... shown in Fig 1 C D ...

The following observations supported the belief that the units were ... in origin

1 Correlation with sympathetic massdischarges As seen in Fig 2 ... appeared together with bursts of mass discharges with the ... of sympathetic activity in human cutaneous nerves as described by ... (1972) Typically the units were activated by manoeuvres k... sympathetic outflow in skin nerves such as a rapid deep b... tion (Fig 3 7) ...

2 *Non defined receptive fields* By contrast with afferent ... ...

TABLE I

Recording site		Unit	Mean firing frequency during rest imp/min	Responses to manoeuvres		
				Talk	Stress	Noise
Median nerve	wrist	1 y 2 z	8 3*	+*	+*	+*
		3 x	—	+	+	+
	elbow	4	9	+	+	+
Peroneal nerve	knee	5	15	+	+	—
		6	—	+	+	+
		7	—	—	(+)	—
	ankle	8	—	—	(+)	—

* indicates sum of impulses in y and z

defined receptive fields but could be activated by various natural or electrical stimuli applied anywhere on the body

3 *Latency of reflex responses* The units appeared in reflex responses to various stimuli at long latencies exceeding 0 5 s in a median nerve recording at wrist level (Fig 1 A B) and exceeding 0 8 s in peroneal nerve recordings at knee level

4 *Effects of Lidocaine blockings* Unitary spikes appearing either as single discharges or in bursts of repetitive impulses remained after total blocking of afferent impulses with 1 % Lidocaine just distal to the recording electrode (Fig 2) but were abolished by a block proximal to the recording site

5 *Collision tests* Collision effects or transient increases in latency of the centripetally conducted single unit responses to electrical stimulation in the skin were sometimes observed in association with spontaneous sympathetic discharges or reflex activation of the sympathetic outflow These findings indicated that the electrical stimulation elicited antidromic impulses in sympathetic fibres interfering with the efferent activity (for details on identifying sympathetic impulses in the electrically induced response see Hallin and Torebjork 1974)

6 *Conduction velocity* Using the collision method conduction velocities were estimated in distal segments of 6 sympathetic fibres Two units recorded at wrist level in the median nerve (Fig 1 A) had conduction velocities of approximately 1 0 and 0 93 m/s (conduction distance 14 cm) Four units in one recording from the peroneal nerve at knee level had conduction velocities of approximately 0 67 0 61 0 59 and 0 58 m/s (conduction distance 36 cm)

7 *Correlation with effector organ responses* Activity in some of the units was correlated with changes in galvanic skin resistance (Fig 7)

Pain	Tapping	Max no of imp in a reflex burst	Max inst frequency imp/s	Corr to GSR	Corr to plethys mogram	Cond velocity m/s
+*	+*	9*		+*	(±)*	1 0 0 93
+	+	7	35	+	(±)	
+	(+)	4	30			
+	+	4	20			0 61
+	–	3	20			
(+)	–	1	2			
–	–	1	1			

Firing patterns of sympathetic units

Table I summarizes some characteristics of eight sympathetic units

Activity during resting conditions' (spontaneous activity) Four units (three in the median nerve and one in the peroneal nerve) were spontaneously active with single discharges occurring sporadically at irregular intervals There were silent periods lasting up to half a minute Occasionally short bursts of 2—3 unitary discharges appeared without obvious external stimuli and the instantaneous firing frequency of a unit could then rise up to 20 imp/s The mean frequency over periods of 2—10 min did not exceed 15 imp/min No correlation was observed between the unitary discharges and the pulse rhythm The coupling with shallow respiration was poor or absent (Fig 3 A)

The remaining 4 units (one in the median nerve and three in the peroneal nerve) were silent for long periods (5—10 min) during resting conditions but they sometimes participated with a single discharge in occasional intense bursts of sympathetic mass activity

Effects of mental activation (talk and stress) When the subjects talked units active during resting conditions exhibited an increased discharge (Fig 4 B F) and in addition previously silent units started firing (Fig 4 F) A tendency for synchronization of discharges in different units into groups was observed

The increase in sympathetic activity was much more pronounced in association with mental stress (Fig 2 B 3 C 4 C F) All 8 units were active and 6 of them fired repeatedly in irregular bursts which were most intense during the first 10—20 s of a stress period (Fig 7) Instantaneous frequencies as high as 35 imp/s were observed and up to 70 impulses occurred in a single unit during the first minute of a stress period Two units (in the peroneal nerve) which were almost complete

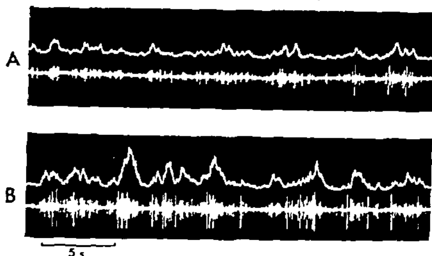


Fig 2 Sympathetic activity recorded from the median nerve at elbow level after Lidocaine block of afferent impulses distal to the recording electrode Discharges in a single unit were often correlated with bursts of multifibre discharges in the original neurogram (lower traces) Integrated neurogram (time constant 0 1 s) shown in upper traces A Resting conditions B Mental stress (*subject threatened with a painful stimulus*)

ly silent during resting conditions and normal conversation discharged sporadically at instantaneous frequencies not exceeding 2 imp/s during stress (Fig 3 C) and the number of discharges during the first minute of stress did not exceed 6

Responses to other manoeuvres Rapid inhalations unexpected loud noises or painful electrical or natural stimuli in the skin elicited bursts of 2—5 (occasionally 7) discharges in 6 units (Fig 4 D) with maximal instantaneous frequencies up to 35 imp/s Two units in the peroneal nerve responded with only a single discharge or not at all to such stimuli (Fig 3 B) Painful or intense stimuli were not necessary to induce reflex activity Touching the skin could induce reflex discharges in 5 units In general the more unexpected and startling a stimulus was the more units were recruited in the reflex responses and the higher was the repetition rate of impulses in each unit (Fig 4 E)

Different recruitment thresholds of individual units The pattern of recruitment was studied in some detail in one recording from the median nerve (illustrated in Fig 1 and 4) where three sympathetic units were identified by collision tests (Hallin and Torebjörk 1974) Two of these units y and were sometimes difficult to differentiate from each other due to similarities in potential waveforms (Fig 1 C) but they were easily distinguished from discharges in a unit x of large amplitude (Fig 1 D) *In the unit analysis the sum of discharges in x* was compared with the sum of discharges in y and together

y and z were spontaneously active with few impulses during resting condition whereas *x* was silent (Fig 4 A) When talking to the subject y and z *sometimes* appeared grouped together (Fig 4 B) and with increasing mental activation *x* also started *firing* typically as the leading unit in groups of discharges from two or three

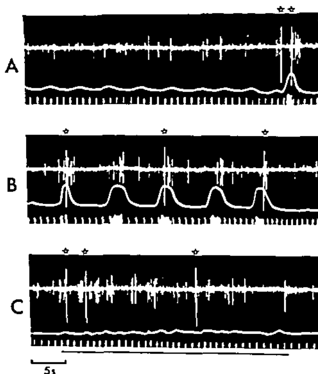


Fig 3 Few unit recording from the peroneal nerve at knee level Upper trace neurogram middle trace respiratory movements (inspiration upwards) lower trace ECG (sometimes disturbed by movement artefacts) A Spontaneous activity in several units without obvious correlation to ECG or respiration during resting conditions (left and middle parts) Note one discharge in a large unit (asterisk) when the subject was instructed to take a deep breath and another discharge associated with the inspiration (right) B Sympathetic bursts accompanying rapid deep inhalations The large unit was not always activated by these manoeuvres C Mental calculation (indicated by bar) increased the sympathetic activity but the firing frequency of the large unit was low

units (Fig 4 C) In reflex responses to painful pinching in the skin (Fig 4 D) or nonpainful unexpected tapping on the skin (Fig 4 E) such groups of impulses appeared in close succession almost superimposed on each other

The mean number of impulses in these units increased from 8 3 imp/min during rest to 19 1 imp/min during conversation and 88 imp/min during stress (Fig 4 F) The increasing proportion of impulses in *x* as compared with the rest of the unit discharges when the subject was activated by increasing mental stimulation is shown in Fig 4 G The percentage of impulses in *x* of all of the impulses in the three units was 0 % during rest 14 % during conversation and 29 % during stress

Reflex responses to electrical stimulation The reflex responses in units *x* *y* and to repeated painful electrical stimulation at constant intensity are shown in Fig 5 The stimulation was performed through intradermal needles in the hand contralateral to the recording site in order to avoid reductions of the reflex responses by collision effects (Hallin and Torebjork 1974) Examples of the responses are shown in Fig 5 C Typically the large unit *x* appeared with 1—4 (occasionally 6) discharges first in the responses followed by several discharges in *y* and The latencies to the first discharges in *x* varied from 530 to 690 ms and the duration of the total response in the three units did not exceed 620 ms The number of unitary discharges in *x* (black bars) and *y* and together (white bars were counted in the time inter

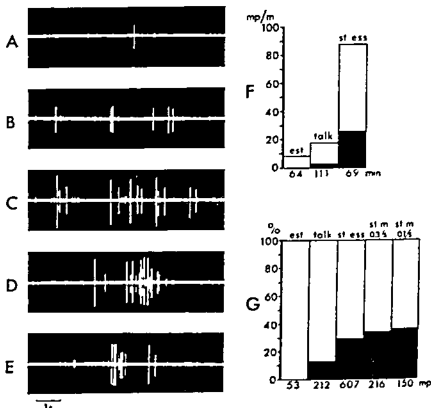


Fig 4 Recruitment of the three units "x" "y" and "z" shown in Fig 1 A Resting conditions Occasional discharges in "y" or "z" B Subject talking Discharges in "y" and "z" were sometimes grouped together C Stress (subject expecting a painful stimulus) Grouped discharges in "x" "y" and "z" D Pinching the skin Groups of discharges occurring in close succession. E Unexpected tapping on the skin Repetitive firing at high frequency in each unit F Mean discharge frequency (imp/min) in "y" and "z" together (white bars) and "x" black bars during several periods of rest, talking and mental stress Total analysis time indicated below bars G Ratio of impulses in "x" (black bars) in percent of total number of discharges in the three units during periods of rest talking and mental stress and in reflex responses to painful electrical stimulation at 0.3 and 0 1 Hz. Total number of impulses indicated below bars

val between 500 and 1350 ms after each stimulus artefact Successive reflex responses to stimulation at 0 3 Hz and 0 1 Hz are shown in Fig 5 A and B respectively As seen in A the intense response to the initial shock was followed by successively decreasing responses as the subject got used to the stimulation Considerable variations in intensity of the reflex responses were observed When the subject's attention was directed towards the stimulation (first horizontal bar in Fig 5 A) a slight increase seemed to occur in the reflex responses whereas a reverse effect was traced when the subject was distracted from the painful shocks (second horizontal bar) A more definite effect of attention on the reflex responses than this has previously been observed in recordings of sympathetic massactivity (Hagbarth *et al* 1972) The reflex responses remained more intense when the stimulation frequency was lower

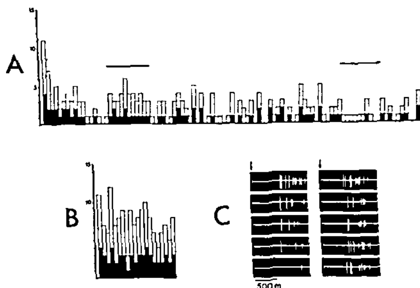


Fig 5 Reflex responses in the median nerve to painful electrical stimulation delivered to the hand contralateral to the recording side A Successive reflex responses at a stimulus frequency of 0.3 Hz Number of impulses/reflex response (indicated to the left) in x (black bars) and $y + x$ (white bars) Left horizontal line marks period when the subject's attention was directed towards the stimuli and right horizontal line indicates period when the subject was instructed not to pay attention to the stimuli B Successive reflex responses at a stimulation frequency of 0 1 Hz (from Hallin and Torebjork 1974) C Examples of reflex responses to stimuli delivered at a frequency of 0 3 Hz (left row) and 0 1 Hz (right row)

(Fig 5 B) Although variations were observed from one reflex response to another in the relationship between impulses in the large unit and the small units together the mean ratio was about 1 2 during both stimulation periods (Fig 4 G)

Suppression of sympathetic activity A suppression of sympathetic activity was observed during the first 1—2 s after the reflex responses to painful electrical stimulation at 0 1 Hz in the median nerve (Fig 6 A) A weak suppression was also observed in one recording from the peroneal nerve The suppression was not accompanied by total inexcitability since reflex responses could be elicited during that period (Fig 6 B)

Sympathetic unit activity correlated with effector organ responses Changes in galvanic skin resistance (GSR) and pulse plethysmogram were measured in the receptive field (third finger) and correlated with activity in 3 sympathetic units recorded from the median nerve Representative examples of the results are shown in Fig 7 Both induced and spontaneous bursts of unitary activity were regularly followed by a slow change in GSR with a latency of the order of 0 7—1 s (Fig 7 A) Unitary bursts in close succession were accompanied by complex GSR responses (Fig 7 B) Mental calculation which was a potent stimulus for prolonged irregular sympathetic discharges was accompanied by complex chan

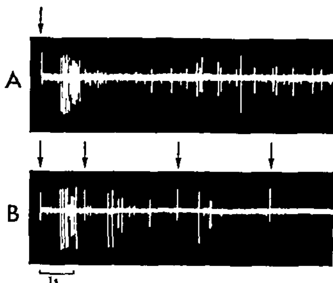


Fig 6 A 10 superimposed reflex responses to painful stimuli delivered at 0.1 Hz. Note silent period of sympathetic activity following the reflex response B Electrical stimuli (arrows) eliciting a reflex response in the period of relative supression

skin resistance (Fig 7 C) However occasional single spikes induced no obvious GSR responses

Pulse plethysmography changes did not show a similar good correlation with the unitary bursts Some representative examples are shown in Fig 7 D Intense bursts evoked by external stimuli were generally followed by slow decreases in the amplitude of the plethysmogram appearing at a latency of about 1 s and lasting for 5—15 s Weak unitary bursts did not induce significant changes and sometimes a decrease in amplitude of the plethysmogram was seen without previous activity in the sympathetic units under study

Discussion

Differentiation between sympathetic activity and sustained firing in afferent fibres
Several types of afferent units supplying mammalian skin exhibit a sustained firing which in recordings from intact cutaneous nerves might be misinterpreted as being activity from sympathetic nerve fibres Iggo s type II mechanoreceptor (Chambers and Iggo 1967) and some thermosensitive C receptors (Hensel Iggo and Witt 1960 Iriuchijima and Zotterman 1960) carry a sustained discharge of impulses dependent on the skin temperature and bursts of impulses may arise from cold receptors with myelinated fibres (Iggo 1964) Pulse synchronous barrages have been reported from Pacinian corpuscles (Gammon and Bronk 1935) and hair follicle receptors innervating tissues close to arterial walls (Hunt and McIntyre 1960 Brown and Iggo 1967) Afferent units similar to Iggo s type II mechanoreceptors and Pacinian receptors have been identified also in human skin nerves (Knibestol and Vallbo 1970) as well as occasional C afferents with a sustained discharge (Torebjork 1974)

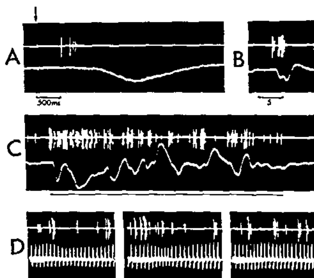


Fig 7 Sympathetic unit activity (upper traces) and effector organ responses (lower traces) A Sudden noise (arrow) elicited a reflex response which was followed by a slow decrease in GSR measured in the innervation zone of the impaled fascicle (pulp of third finger) B Repeated bursts succeeded by complex GSR changes C Mental calculation (bar) inducing dramatic increase in unit activity accompanied by complex changes in GSR D Examples of unit activity and plethysmographic changes in pulse amplitude in the tip of the middle finger Bursts of sympathetic activity were sometimes followed by slow changes in pulse amplitude Time calibration identical in B—D

Careful testing with natural stimuli within the receptive skin field of the explored fascicle will evoke characteristic neural responses which permit a reliable classification of afferent units By contrast the sympathetic impulses can be induced not only from the innervation zone of the impaled fascicle but also by stimuli outside this area (Hallin and Torebjörk 1970) In this context it should be considered that manoeuvres increasing the sympathetic outflow could induce pilomotor or vasomotor effects in the skin which in turn might elicit afferent activity (Nilsson 1969) possibly mimicking sympathetic reflex responses The results of the Lidocaine blockings (Fig 2) established that the neural signals under study were of efferent character Such blockings were not necessary to repeat in each recording since the firing pattern of the units described in this report with single or repetitive impulses appearing in association with multifibre bursts of discharges previously identified as sympathetic (Hagbarth *et al* 1972) was so characteristic that it could be discriminated from signals in afferent fibres In addition the results of manoeuvres known to increase the sympathetic outflow in human skin nerves (Hallin and Torebjork 1970 Hagbarth *et al* 1972 Delius *et al* 1972 c), the collision tests (Hallin and Torebjork 1974) the conduction velocity determinations and the correlation of neural impulses with effector organ responses (Fig 7) lend credence to the conclusion that the unit activity described in this report derived from postganglionic sympathetic fibres

Firing pattern of sympathetic units in cutaneous nerves The unit material is too small to establish firm conclusions as to typical discharge frequencies of sympathetic units in human cutaneous nerves It is evident that there is spontaneous firing at very low frequency in some units during resting conditions whereas others are silent The spontaneous firing was irregular and did not show any apparent cor

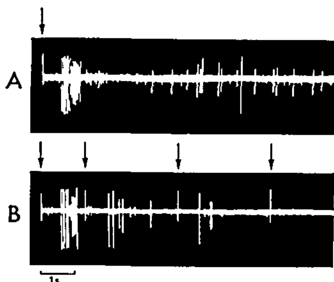


Fig. 6. A. 10 superimposed reflex responses to painful stimuli delivered at 0.1 Hz. Note silent period of sympathetic activity following the reflex response. B. Electrical stimuli (arrows) eliciting a reflex response in the period of relative supression.

skin resistance (Fig. 7 C). However, occasional single spikes induced no obvious GSR responses.

Pulse plethysmography changes did not show a similar good correlation with the unitary bursts. Some representative examples are shown in Fig. 7 D. Intense bursts evoked by external stimuli were generally followed by slow decreases in the amplitude of the plethysmogram appearing at a latency of about 1 s and lasting for 5—15 s. Weak unitary bursts did not induce significant changes and sometimes a decrease in amplitude of the plethysmogram was seen without previous activity in the sympathetic units under study.

Discussion

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reflex activation through different spinal or supraspinal routes cannot be excluded (Sato 1972 a)

Intense reflex responses with repetitive firing in the units were followed by a silent period of the sympathetic activity for 1—2 s This phenomenon, described in sympathetic preganglionic neurons by Pitts and Bronk (1942) has partly been attributed to post excitatory depression (subnormality) of the preganglionic neuron itself (Polosa 1967) but there is also evidence of a somatic afferent inhibitory process in the spinal cord (Beacham and Perl 1964 Sato 1972 b) and in particular descending inhibitory effects from supraspinal levels (i e Pitts and Bronk 1942 Koizumi *et al* 1968 Iwamura *et al* 1969 Sato 1972 b) The possibility that inhibitory processes contribute to the sudden ending of the reflex burst after a single stimulus has been pointed out (Beacham and Perl 1964) and such suppression which is most pronounced during the first 0 1—0 2 s after a sympathetic reflex volley (Schmidt and Schonfuss 1970) would probably contribute to the pattern of pauses in between sympathetic bursts in both cutaneous and muscle nerves It is also conceivable that the decrease in reflex responses to electric shocks applied at intervals of about 3 s (Fig 5 A) or less were due not only to the fact that the subject became used to the stimulation but also to inhibitory aftereffects dependent on the stimulation frequency (Schmidt and Schonfuss 1970) In one experiment there was a rather constant mean ratio between impulses in one unit as compared with impulses in two other units in reflex responses to electric shocks at various frequencies (Fig G see also Hallin and Torebjörk 1974) Possibly not only synchronized activation of the units but also common suppression phenomena influenced this relationship

Sympathetic unit activity and effector organ responses It is well known that blocking of the sympathetic outflow to the extremities in man is followed by an increased circulation and warming of the skin (Brown and Adson 1925) due to released vasoconstrictor tone Marked constrictor responses of the cutaneous vessels to the paws of the cat were elicited by sympathetic stimulation at frequencies as low as 15 imp/min (Celander and Folkow 1952) This indicates that the observed spontaneous firing in some sympathetic units during resting conditions (mean rates up to 15 imp/min) may have physiological significance Some of the units probably had a sudomotor function since the unit activity was well correlated with changes in GSR whereas the correlation with plethysmographic signs of skin vasoconstriction within the innervation zone was less convincing (Fig 7) However the manoeuvres used were not particularly well suited to differentiate between sudomotor and vasomotor outflow since not only GSR changes but also vasoconstrictor changes are known to occur after the stimuli used (for references see Greenfield 1963) In future studies controlled changes in temperature would be preferable for inducing more selective action of sudomotor and vasomotor fibres From the present results it cannot be decided whether the two high threshold units in the peroneal nerve might innervate different effector organs than the rest of the unit population or if their particular discharge pattern might be due to a different innervation

relation with the pulse rhythm and a synchronization with normal respiration was only occasionally observed This accords with results from multiunit recordings of sympathetic activity to the skin in both cat (Ninomiya et al 1973) and man (Hagbarth et al 1972) indicating that the major part of the sympathetic activity to the skin is baroreceptor independent As pointed out by Widdicombe (1966) and Green and Heffron (1968) there might exist rhythmic firing in sympathetic units which are not revealed by simple inspection of the neurogram In the absence of more sophisticated analytical methods (cf Mannard and Polosa 1973) we cannot exclude the possibility of undetected rhythmic firing in sympathetic units in human cutaneous nerves

A consistent finding in all of our recordings was the marked influence of mental activity upon the sympathetic outflow to the skin As seen in Fig 4 F there was a tenfold increase in the total number of impulses/min in three units during periods of mental stress as compared with resting conditions The increase in sympathetic outflow was due to both an increase in firing frequency of previously active units and recruitment of previously silent fibres As originally shown by Pitts Larrabee and Bronk (1941) the sympathetic system functions in this respect similarly to other efferent systems to produce a graded effector response

The sympathetic units in the experiment illustrated in Fig 4 C exhibited a tendency for coactivation in groups of impulses when the attention of the subject was raised This is in accordance with previous anatomical and physiological findings indicating a synchronization of impulses in sympathetic fibres both at preganglionic (Bronk et al 1936) and ganglionic (Billingsley and Ranson 1918 Adrian et al 1932) levels On further increase in attention and in reflex responses to unexpected stimuli the groups of unitary impulses appeared in close succession forming bursts of discharges probably as an indication of repeated preganglionic activation The number of repetitive impulses in close succession in a burst generally did not exceed 5 (occasionally 7) a figure consistent with up to 5 repetitive impulses recorded in preganglionic unit bursts in intact cats in response to an acoustic click stimulus (Iggo and Vogt 1960) Also the maximal instantaneous frequency of a unit in bursts of impulses up to 20/s during resting conditions and 35/s during stress is compatible with their figure of 30/s in preganglionic fibres in the undisturbed cat The independent firing of the units during resting conditions (Fig 4 A) and the variability in the pattern of the grouped discharges when two or three units appeared together (Fig 4 B—C) indicates that these three units were activated by more than one preganglionic fibre

The reflex discharges were probably induced by afferent A fibre activation (cf Janig et al 1972) since even weak touch stimuli to the skin could elicit the reflex responses and the latencies to the first impulses in the reflex discharges were too short to be consistent with activation by afferent C fibres In some experiments as shown in Fig 1 the reflex discharges in one unit had already ceased when the discharges in other units had begun This could be due to different conduction velocities in preganglionic (Janig and Schmidt 1970) or postganglionic fibres but

HAGBARTH K E and A B VALLBO Pulse and respiratory grouping of sympathetic impulses in human muscle nerves *Acta physiol scand* 1968 *74* 96—108

HALLIN R G and H E TOREBJORK Afferent and efferent C units recorded from human skin nerves in situ *Acta Soc Med upsalien* 1970 *75* 277—281

HALLIN R G and H E TOREBJORK Methods to differentiate electrically induced afferent and sympathetic C unit responses in human cutaneous nerves *Acta physiol scand* 1974 *92* 318—331

HENSEL H, A IGGO and I WITT A quantitative study of sensitive cutaneous thermoreceptors with C afferent fibres *J Physiol* (Lond) 1960 *153* 113—126

HUNT C C and A K MCINTYRE An analysis of fibre diameter and receptor characteristics of myelinated cutaneous afferent fibres in cat *J Physiol* (Lond) 1960 *153* 99—112

IGGO A. Temperature discrimination in the skin *Nature* (Lond) 1964 *204* 481—483

IGGO A and M VOGT Preganglionic sympathetic activity in normal and in reserpine treated cats *J Physiol* (Lond) 1960 *150* 114—133

IRIUCHIJIMA J and Y ZOTTERMAN The specificity of afferent cutaneous C fibres in mammals *Acta physiol scand* 1960 *49* 267—278

IWAMURA Y, Y UCHINO S OZAWA and N KUDO Excitatory and inhibitory components of somato-sympathetic reflex *Brain Res* 1969 *16* 351—358

JANIG W, A SATO and R F SCHMIDT Reflexes in postganglionic cutaneous fibres by stimulation of group I to group IV somatic afferents *Pflugers Arch ges Physiol* 1972 *331* 244—256

JANIG W and R F SCHMIDT Single unit responses in the cervical sympathetic trunk upon somatic nerve stimulation *Pflugers Arch ges Physiol* 1970 *314* 199—216

KNIBESTOL M and A B VALLBO Single unit analysis of mechanoreceptor activity from the human glabrous skin *Acta physiol scand* 1970 *80* 178—195

KOIZUMI K, A SATO A KAUFMAN and C McC BROOKS Studies of sympathetic neuron discharges modified by central and peripheral excitation *Brain Res* 1968 *11* 212—224

MANNARD A. and C POLOSA, Analysis of background firing of single sympathetic preganglionic neurons of cat cervical nerve *J Neurophysiol* 1973 *36* 398—408

NILSSON B Y Structure and function of the tactile hair receptors on the cat s foreleg *Acta physiol scand* 1969 *77* 396—416

NINOMIYA I A IRISAWA and N NISIMARU Nonuniformity of sympathetic nerve activity to the skin and kidney *Amer J Phys ol* 1973 *224* 256—264

PITTS R F and D W BRONK Excitability cycle of the hypothalamus-sympathetic neurone system. *Amer J Physiol* 1942 *135* 504—522

PITTS R F M G LARRABEE and D W BRONK An analysis of hypothalamic cardiovascular control *Amer J Phys ol* 1941 *134* 359—383

POLOSA C The silent period of sympathetic preganglionic neurons *Canad J Physiol Pharmacol* 1967 *45* 1033—1045

SATO A. The relative involvement of different reflex pathways in somato-sympathetic reflexes analyzed in spontaneously active single preganglionic sympathetic units *Pflugers Arch ges Physiol* 1972 a. *333* 70—81

SATO A Spinal and supraspinal inhibition of somato-sympathetic reflexes by conditioning afferent volleys *Pflugers Arch ges Physiol* 1972 b *336* 121—133

SCHMIDT R F and K SCHONFUSS An analysis of the reflex activity in the cervical sympathetic trunk induced by myelinated somatic afferents *Pflugers Arch ges Phys ol* 1970 *314* 175—198

TOREBJORK H E Afferent C units responding to mechanical thermal and chemical stimuli n human non glabrous skin *Acta physiol scand* 1974 *92* 374—390

TOREBJORK H E and R G HALLIN Identification of afferent C units in intact human skin nerves *Brain Res* 1974 *67* 387—403

VALLBO A B and K E HAGBARTH Activity from skin mechanoreceptors recorded percutaneously in awake human subjects. *Exp Neurol* 1968 *21* 270—289

WALLIN B G W DELIUS and K E HAGBARTH Comparison of sympathetic nerve activity in normotensive and hypertensive subjects *Circulat Res* 1973 *33* 9—21

WIDDICOMBE J G Action potentials in parasympathetic and sympathetic efferent fibres to the trachea and lungs of dogs and cats *J Physiol* (Lond) 1966 *186* 56—88

Acta physiol. scand. 1974. 92. 318—331

From the Department of Clinical Neurophysiology, University Hospital, Uppsala, Sweden

Methods to Differentiate Electrically Induced Afferent and Sympathetic C Unit Responses in Human Cutaneous Nerves

By

ROLF G. HALLIN and H. ERIK TOREBJÖRK

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Abstract

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C unit responses to intradermal electrical stimulation were recorded with tungsten microelectrodes inserted percutaneously into intact human cutaneous nerves. An increase in discharge frequency was associated with a decrease in conduction velocity in unmyelinated fibres and this phenomenon was used to identify *afferent* C units which responded to both electrical and natural stimuli in the skin. Antidromic impulses in *efferent* sympathetic fibres were also elicited by electrical stimulation in the skin as judged by latency changes or signs of collisions in the C responses associated with activity in sympathetic fibres. In this way conduction velocities in distal segments of both afferent and sympathetic C fibres can be estimated. Furthermore the possibility to differentiate afferent and sympathetic C units is of obvious importance for the study of their respective discharge characteristics and in psychophysiological studies in alert man.

In conventional recordings of single unit activity from animal nerves the number of active fibres adjacent to the recording electrode is usually reduced by dissecting the nerve trunk into fine filaments. This dissecting technique has been used also in recordings from human nerves (Hensel and Boman 1960) but it has for obvious reasons limited application in man. With the use of microelectrodes it is possible to record multiunit activity in both A and C fibres in intact human cutaneous nerves (Hallin and Torebjörk 1970 a) but in these recordings it is sometimes difficult to determine from signals in the original neurogram if receptors with myelinated or nonmyelinated fibres respond to a natural stimulus in the skin. In particular the identification of single C unit discharges may be difficult especially when the potentials of different units have similar amplitudes and interfere with each other. Nevertheless, it has been shown that single C unit impulses can be discriminated in recordings from intact human nerves (Torebjörk and Hallin 1970) and discharge

characteristics of receptors supplied by afferent C fibres in man have been described (Hallin and Torebjork 1970 b van Hees and Gybels 1972 Torebjork and Hallin 1974 a) In addition single sympathetic C units were identified in reflex responses induced by electrical stimuli and various manoeuvres which arouse the attention of the subject (Hallin and Torebjork 1970 b)

The C unit potentials are generally best identified in responses to electrical stimulation especially when the conduction distance is long due to temporal dispersion of impulses in fibres with different conduction velocities On repeated intradermal stimulation the C unit responses exhibit characteristic latency changes depending on the firing frequency (Torebjork and Hallin 1974 b) We suggested that slowing of the impulse propagation in thin nerve fibres during repetitive excitation by combined electrical and natural stimuli might be used to improve the identification of those afferent C units which respond to natural stimulation in the skin and to differentiate between afferent and efferent C units

The purpose of this paper is to present evidence that not only afferent C fibres but also efferent sympathetic fibres are excited by electrical stimulation in the skin Methods to identify and differentiate between discharges in afferent and sympathetic units in centripetally conducted C fibre responses in intact human nerves will be described on the basis of a few illustrative recordings Additional C unit investigations where these methods were applied will be presented in subsequent reports (Hallin and Torebjork 1974 b Hallin 1974 Torebjork 1974) Parts of the present results have appeared previously (Hallin and Torebjork 1974 a)

Methods

Subjects The subjects were two males aged 25 and 30 years with no signs or symptoms of any neurological or cardiovascular disease Both subjects were familiar with the experimental situation

General procedure During the experiments the subjects sat relaxed in a semivertical position with the extremities comfortably supported in stable positions Room temperature varied between 22 and 25 C

Material Two illustrative recordings are presented in this paper but we have verified the general principles in more than 50 single C unit recordings from different cutaneous nerves One recording was made from the peroneal nerve about 2 cm distal to the fibular head (afferent units in Fig 1—2) and the other from the median nerve 2 cm proximal to the radiocarpal joint (sympathetic units in Fig 4—6) Tungsten electrodes were inserted into sensory nerve fascicles supplying hairy skin of the lateral calf and glabrous skin on the volar part of the third digit respectively The innervation zones were determined by the insertion paraesthesias as experienced by the subjects and by the extension of the receptive fields from which neural activity could be evoked by touch stimuli (Vallbo and Hagbarth 1968 Hagbarth *et al* 1970)

Technical details The tungsten microelectrodes and the recording and display system were described in detail previously (Vallbo and Hagbarth 1968 Hagbarth *et al* 1970 Hallin and Torebjork 1973) The full bandwidth of the recording and display system 0 2—10 kHz was used when the amplitude and shape of the unitary potentials were studied The signal-to-noise ratio of the impulses was improved by feeding the signals through a filter with a bandwidth of 0 5—2 kHz and then through an amplitude discriminator adjusted to eliminate about 50—75 of the remaining noise (Hagbarth *et al* 1970) No amplitude calibration of the filtered nerve signals will be presented since the impulses were distorted by filtering In studying the effects of repeated electrical stimulation on the C responses a compact display as illustrated in Fig 6 A was sometimes used Details concerning this display were described in a previous report (Torebjork and Hallin 1974 b)

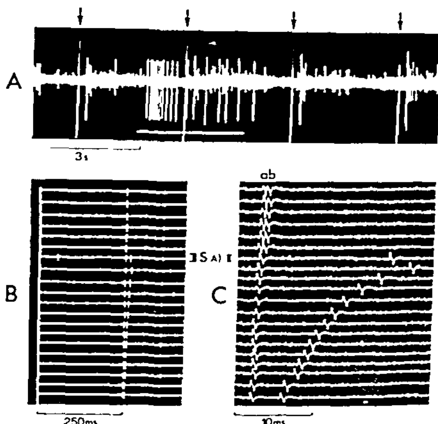


Fig 1 C unit activity recorded in the peroneal nerve 2 cm distal to the fibular head. Electrical stimuli arrows applied every three seconds to the receptive skin area laterally on the calf Conduction distance 25 cm. A Unitary responses to a combination of electrical stimulation and a pinprick bar 3 mm proximal to the stimulating electrodes. B Successive C responses to electrical stimulation Note changes in latency for one component in the responses associated with the unit activity evoked by natural stimulation in A, S A C Same sequence as B at a shorter time base with the first 245 ms after stimulus artefacts omitted *a* and *b* indicate two different C units

Stimulation of C units The extent of the receptive fields from which neural activity could be evoked was estimated by strokings with emery cloth Testing with von Frey's hairs needle pricks or thermal radiation from heated metal rods were used to induce C unit discharges from the skin

For electrical stimulation a DISA stimulator unit (Type 14 E 01) was used, which delivered rectangular voltage pulses with a duration of 0 05—0 2 ms and an amplitude of 100—500 V The stimulation frequency varied from 0 1 to 5 Hz. The stimulating electrodes were 2 small steel needles approximately 0.3 mm in diameter inserted intradermally about 3—8 mm apart within the skin area supplied by the impaled fascicle Repeated adjustments of stimulating and recording electrodes were performed until C unit activity was identified in the neurogram in responses to the electric shocks

Reflex activity in sympathetic fibres was induced either by different manoeuvres arising the attention of the subject, such as a loud noise or mental calculation or the subject was instructed to take a rapid deep breath Such stimuli are known to induce pronounced sympathetic reflex responses in human cutaneous nerves (Hallin and Torebjork 19 0 b Hagbarth *et al* 1972 Delius *et al* 19 2

Care was always taken that the stimulating electrodes were not displaced during natural stimulation in the skin, and that no muscle artefacts were induced by the electric stimuli or by startle reactions to manoeuvres eliciting sympathetic reflex responses.

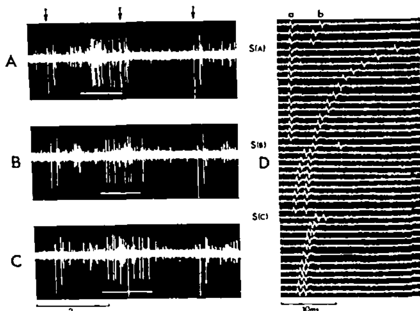


Fig 2 Activation of afferent C units with adjacent receptive fields Same recording as in Fig 1 Unitary responses to combined electrical stimulation (arrows) and needle pricks (bars) A 3 mm proximal to B 3 mm distal to and C between the stimulating electrodes in the skin D Continuation of sequence C in Fig 1 (first three responses identical with last responses in Fig 1 C) S (A) S (B) and S (C) refer to natural stimuli in A B and C respectively

Results

Afferent C unit responses induced by combined electrical and natural stimuli In the peroneal nerve recording illustrated in Fig 1 and 2 two C units *a* and *b* with amplitudes of about 40 μV appeared together along with several C components of lower amplitudes at a latency exceeding 250 ms in responses to electric shocks delivered once every 3 s to the lateral calf (Fig 1 B) Natural stimuli were also applied to the skin area which was simultaneously subjected to electrical stimulation A needle prick about 3 mm proximal to the stimulating electrodes (area 1 in Fig 3 A) elicited unit discharges of similar amplitude as the C units (Fig 1 A) and at the same time the latency of the electrically induced responses in unit *b* increased by 16 to 20 ms (Fig 1 C) After terminating the natural stimulus the latencies of the electrically induced responses in unit *b* successively decreased in the following minute (Fig 1 C bottom) Repeated testing in the same skin area gave similar results (Fig 2 A D top) The other C components were relatively uninfluenced by these needle pricks as judged by their basically unchanged latencies in the responses to electrical stimulation (Fig 1 B 2 D top)

A sparse unitary activity of a similar kind as that seen before was provoked by needle pricking a few mm distal to the stimulating electrodes (area 2 in Fig 3 A

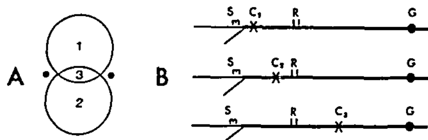


Fig 3 Schematic drawings illustrating A the test situations corresponding to Fig 1 and 2 and B the collisions illustrated in Fig 4 and 5 A Dots mark sites of intradermal stimulating electrodes at interelectrode distance of 5 mm Unit *b* was activated by natural stimuli within area 1 *unit a from area 2 and both units from area 3* B *Arrangement of stimulating (S)* and recording electrodes (R) during collision tests in sympathetic fibres G ganglion C_1 and C_2 represent collisions distal to recording electrodes and C_3 collision proximal to recording site

but in this case an abrupt increase in latency of unit *a* was observed (Fig 2 B D middle) The successive decrease in latency of unit *b* following a previous test was not affected

A needle prick applied to a skin area between the stimulating electrodes (area 3 in Fig 3 A) also induced unit activity (Fig 2 C) and this time both unit *a* and *b* exhibited an increase in latency and slow recovery phases after the testing (Fig 2 D bottom)

There were no signs of indirect mechanical movements of the stimulating electrodes on testing with the superficial needle pricks and similar results as above were obtained after readjustment of the stimulating electrodes (increasing the interelectrode distance from 5 to 7 mm)

In this recording bursts of efferent sympathetic massdischarges with the general characteristics described by Hagbarth *et al* (1972) were identified (note irregular increases in background activity in Fig 1 A 2 A—C) No changes in latency of the responses in units *a* and *b* were observed in association with such sympathetic bursts

Sympathetic C unit discharges induced by electrical stimulation Reflex responses in sympathetic fibres to intradermal electrical stimulation appear with variable intensities at long varying latencies (exceeding 0 5 s in the median or radial nerves at wrist level) and can be differentiated from centripetally conducted C responses both in multiunit recordings (Torebjork and Hallin 1973) and in single unit recordings (Hallin and Torebjork 1970 b) The question remains whether antidromic impulses in efferent sympathetic fibres contribute to the centripetally conducted C responses to electric stimulation in the skin As will be shown such electrically induced antidromic sympathetic impulses can be identified by *A* collision tests and *B* demonstration of latency changes in the centripetally conducted C unit responses in association with sympathetic outflow

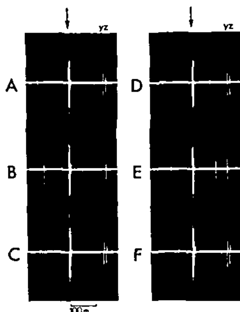


Fig 4 Signs of collisions in sympathetic fibres distal to the recording site on electrical stimulation at 1 Hz in the skin of the third finger Recording from the median nerve at the wrist in Fig 4—6 Conduction distance 14 cm. Stimulus artefacts with A fibre responses indicated by arrows and centripetally conducted responses in two C units indicated by y and z in Fig 4—6 A C D and F are controls In B and E one of the C units is missing in the responses associated with discharges in sympathetic units of similar amplitudes as y and z

A 1 Collisions between orthodromic and antidromic impulses in sympathetic fibres between the stimulating and recording sites Fig 4—6 illustrate sympathetic single unit activity recorded from the median nerve at the wrist of the right hand Superimposed reflex responses to painful electric stimulation (0 1—0 2 Hz) in the skin of the left and right hands are shown in Fig 5 A—B Each reflex response had a latency exceeding 0 5 s and consisted of 1—5 discharges in one large unit x followed by several discharges in smaller units Reflex responses of this type were also elicited by a deep inhalation or a sudden loud noise

Electric shocks delivered to the receptive field (third digit) of the impaled fascicle elicited centripetally conducted responses where two units y and z appeared at latencies of 140 and 150 ms (Fig 4 5 B and 6) The conduction velocities of these units 1 0 m/s and 0 93 m/s respectively were within the C fibre range The amplitudes of these units were similar to those of the smaller units in the sympathetic reflex responses and were also similar to the amplitudes of unit potentials which appeared irregularly at low frequency during periods between bursts of sympathetic activity

No receptive skin areas were found from where activity in units y and z could be directly induced by natural stimuli such as firm pressure or pinpricks and no changes in latency of the type illustrated in Fig 1 and 2 were observed in immediate relation to these stimuli Several times however one or the other of the C units failed in the centripetally conducted responses when the electric stimuli coincided with a period of increased sympathetic activity provoked for instance by a deep inhalation a loud noise or an unexpected mechanical stimulus applied anywhere

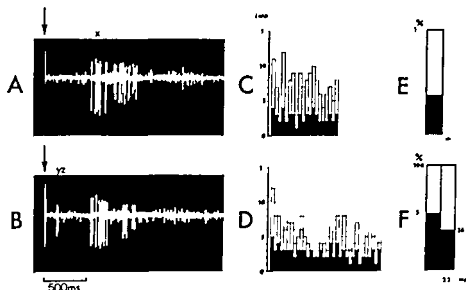


Fig 5 Signs of collisions in sympathetic fibres proximal to the recording site A—B Five superimposed reflex responses to painful electrical stimuli in the receptive field of the impaled fascicle B and in the contralateral hand A Stimulus frequency 0 1 Hz in A 0 2 Hz in B The responses consist of discharges in a large unit x and smaller units Note reduction of the number of discharges in the small units in B collision situation as compared with A control situation C—D Number of discharges in unit x (black bars) and the small units together (white bars) in reflex responses to successive electrical stimuli C refers to "control situation in A and D to collision situation in B Bars included within broken lines in D represent addition of two extinguished impulses in small units to each reflex response E—F Sum of impulses in x (black bars) in % of total number of unitary reflex discharges during control period (C) E and collision period (D) F before (left) and after (right) addition of extinguished impulses in small units Total number of reflex impulses indicated under the bars

on the body of the subject Such failure was also observed when the electric shocks delivered at very low frequency (0 2 Hz) coincided with spontaneous unit discharges between the bursts

Fig 4 illustrates situations when reflex discharges in sympathetic fibres had passed the recording electrodes about 100 ms before (B) and 90 ms after (E) application of the electric shocks An impulse of corresponding amplitude as the sympathetic unit discharge was missing at the expected latency in the C response (compare controls in A C D F) These findings suggested that collisions between antidromic and orthodromic impulses had occurred between the stimulating and recording sites as schematically illustrated in Fig 3 B (top drawing corresponding to Fig 4 B and middle drawing corresponding to Fig 4 E)

A 2 Collisions in sympathetic fibres between the recording site and the ganglion Collisions of orthodromic and antidromic impulses between the recording electrode and the sympathetic ganglion (Fig 3 B bottom) should cause a reduction of the number of unit potentials in the sympathetic reflex responses This is illustrated in Fig 5 On electric stimulation within the receptive field of the impaled fascicle

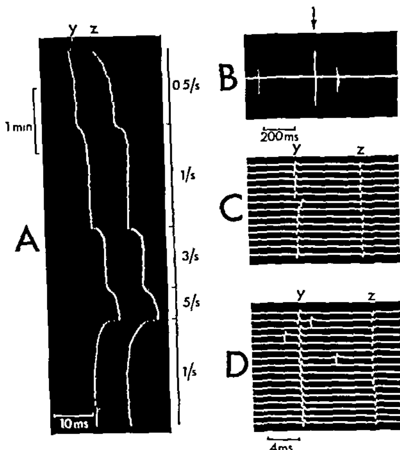


Fig 6 A Effect of electrical stimulation at constant intensity and varying frequencies (indicated to the right) on centripetally conducted responses in sympathetic C fibres Each unit response represented by a dot in the compact display First 130 ms after stimulus artefacts omitted B—C Transient latency changes in unit response y to electrical stimulation at 1 Hz C in association with a discharge in a sympathetic unit of similar amplitude preceeding the stimulus in B D No transient latency changes or blockings in y or and no reflex discharges in small sympathetic units were observed on stimulation at 3 Hz in spite of reflex activation of the large unit x

when the two units y and were directly excited the gap between the large and small unitary discharges in the reflex responses was widened (collision situation Fig 5 B) as compared with reflex responses elicited from the contralateral hand (control situation Fig 5 A) The mean ratio between large and small amplitude discharges in the reflex responses was about 1 2 in the control situation (Fig 5 E) although variations occurred in each reflex response (Fig 5 C compare also Hallin and Torebjork 1974 b) In the collision situation the proportion of small amplitude reflex discharges was reduced and a mean ratio of about 1 1 was found By adding two discharges in small amplitude units to each reflex response the previous relationship between large and small unit impulses was fairly well restored (Fig 5 D F)

B. Latency changes in centripetally conducted C unit responses associated with sympathetic outflow. Repeated intradermal electrical stimulation of C fibres at frequencies as low as 0.5 Hz causes an increase in the latency of the C fibre responses (Torebjörk and Hallin 1974 b). The C units y and z were influenced in a similar way. In Fig. 6 A the centripetally conducted C unit responses are represented as dots and successive responses displayed below each other to present in a compact form latency changes occurring during a long period of stimulation. The stimulus intensity was kept constant and was suprathreshold for both units and the stimulation frequency was varied from 0.5 to 5 Hz. Each rise of the stimulus frequency caused an increase in latency and conversely a reduction of the stimulation frequency resulted in a decreased latency for both units.

After a period of regular stimulation the latencies stabilized at a relatively constant value dependent on the stimulation frequency. In analogy with afferent C units it was expected that additional firing of units y and z elicited by stimuli other than the electric shocks, would induce a transient increase in latency followed by a successive decrease when the additional firing stopped. This is illustrated in Fig. 6 B—C. A transient increase in latency of responses in unit y to electrical stimuli at 1 Hz was observed (C) when a reflex sympathetic discharge of similar amplitude as y preceeded the initiation of the electric shock (B). Latency changes of this kind were seen several times in responses of y or z to electrical stimuli at low frequencies in association with activity in sympathetic fibres.

An increase of the stimulation frequency should increase the possibility for collisions between antidromic and orthodromic discharges. As seen in Fig. 6 D, where the stimulating frequency was 3 Hz, no latency changes or blockings of the responses in y and z were seen in spite of reflex activation of the sympathetic outflow. Only impulses in the large unit x, which was not directly excited by the electrical stimulation, reached the recording site, whereas orthodromic impulses in all small units of the reflex responses were extinguished by collisions proximal to the recording site during periods of high frequency stimulation.

Discussion

I. Identification of afferent units in the C response to intradermal electrical stimulation

A period of repetitive activity in a nerve is followed by aftereffects (Hering 1884) such as a "positive after potential" which is cumulative and dependent on the stimulation frequency and the duration of the stimulation period (Gasser 1935). Responses in nonmedullated C fibres are followed by a particularly marked "positive after potential" (Bishop 1934), which is prolonged following tetanic C fibre stimulation (Grundfest and Gasser 1938). During the "positive after potential" the excitability and the conduction velocity of the fibres are subnormal (Gasser 1935). It has been suggested that the long lasting hyperpolarization in C fibres following tetanic stimulation might be associated with relatively large ionic changes occurring

during repetitive activity in thin fibres having a large surface/volume ratio as compared with myelinated fibres and giant axons (Ritchie and Straub 1957).

In attempting to classify centripetally conducted C unit responses to electrical stimulation as afferent or efferent we took advantage of the observation that intradermal stimulation at frequencies as low as 0.5 Hz provoked increases in latency of unitary C responses recorded from intact human cutaneous nerves (Torebjörk and Hallin 1974 b). In addition the slowing of conduction is most pronounced for the first impulses in a train (Franz and Iggo 1968) and recovery of conduction is also most rapid immediately after a decrease in firing frequency (Fig. 6 A). Even a single additional impulse could produce a visible increase in latency of C unit responses to intradermal electrical stimulation at a frequency of 1 Hz and when the unit occasionally failed to respond a decrease in latency was noted in the next response (Torebjörk and Hallin 1974 b). Thus with constant stimulation at low frequency latency changes in the C responses serve as a sensitive index of changes in firing frequency in the units under study. Furthermore the recovery of the conduction velocity after a period of increased activity is rather slow (Fig. 1, 2). The electrical test shocks can thus be applied at low frequencies (down to 20/min in the illustrated experiment) without missing this typical indication of previous activity in the fibre.

Some limitations and sources of possible errors in identifying afferent C units

1. Displacement of the stimulating electrodes in the skin when testing with mechanical stimuli could induce abrupt changes in latency due to excitation at different sites along the fibres and successive changes in latency indicating increased activity in C fibres could also occur if the electrodes themselves mechanically triggered the receptive elements. Such errors were avoided by exerting great care not to move tre electrodes when testing with natural stimuli. The rest results could also be checked after replacing the stimulating electrodes in different positions in the skin.

2. Changes in temperature are known to affect the conduction velocity in peripheral nerves. Franz and Iggo (1968) showed that each degree drop in temperature produced a 2.17 % reduction in conduction velocity of nonmyelinated axons. Similarly diffuse cooling of the skin around the stimulating electrodes by ether evaporation caused a decrease in conduction velocity in superficial nerve fibre segments whereas a diffuse heating of the skin increased the conduction velocity (Torebjörk 1974). If temperature sensitive receptors were activated by such stimuli the combined effects of slowing of conduction velocity due to increased firing and decrease or increase in velocity due to temperature effects on peripheral nerve conduction would result in complicated latency patterns in the electrically induced responses. Acknowledging these factors which may lead to difficulty in interpretation of the analysis not only mechanical but also thermal (heat) and chemical stimuli can be used to identify active C elements in the electrically induced responses (Torebjörk 1974).

3. Confusion with sympathetic fibres. Different stimuli in the skin can induce reflex responses in efferent sympathetic fibres especially if the stimuli are un

expected (Hagbarth *et al* 1972) Thus the simple association of a skin stimulus *with signs of increased firing in a C fibre responding to electrical stimuli in the same area should not be taken as proof for afferent fibre activation unless it can be* demonstrated that the unit is activated *only from a well-defined receptive field* and that it cannot be activated by *manoeuvres known to elicit reflex activity in* sympathetic fibres (Hallin and Torebjork 1970 b Hagbarth *et al* 1972, Delius *et al* 1972) Intense natural stimuli might induce antidromic impulses in efferent fibres but *we have as yet not observed this phenomenon If it should occur, and the same efferent fibre could not be activated by reflex stimulation then a false classification* could be made

4 Effects of electrical stimulation at the receptors When stimulating through intradermal needles nerve twigs at different depths in the skin are probably excited since electric shocks induced impulses in C fibres also when the superficial receptive endings were depressed by a local anesthetic applied on the skin (*Hallin* 1974) The possibility exists then that collisions could occur between *orthodromic impulses from the receptive endings and antidromic impulses induced proximally* by the electric shocks Since the distal segments where collisions could occur are probably short and since the electrical stimulation frequency is low only occasional *collisions or re settings of the firing rhythm due to interference with antidromic* impulses at the receptor (Matthews 1931) would be expected and these effects *would not appreciably conceal the latency changes in the* C responses during receptor activation It cannot be excluded that the excitability of the C *receptors is in*fluenced by long term stimulation even at low frequencies and hence *qualitative but not quantitative testing of receptors with natural stimuli was performed during* simultaneous electrical excitation

Conclusions The characteristic changes in latency induced by a combination of electrical and natural stimuli serve as a marking of *those* C *units that respond to the natural stimuli whereas elements that are not activated by* the natural stimuli *remain uninfluenced in the electrically induced C responses By comparing* the unit *potentials in responses to natural and electrical stimuli* the accuracy in classification of the signals as deriving from A or C fibres and *from afferent or sympathetic* fibres is improved and the method *is* also valuable in mapping the receptive fields of afferent C units For example a mere inspection of the original neurogram in Fig *1—2 would easily lead to the erroneous conclusion that the* unitary responses to pinprick in different skin areas derived from only one unit However by studying the latency changes of the responses to combined electrical and natural stimuli the *following conclusions could be drawn*

1 The unit activity derived *from* C *fibres* with conduction velocities of approximately *1 m/s*

2 Two different C units responded to the stimuli unit *b* was activated in Fig 2 A unit *a* was activated in Fig 2 B and both units were activated in Fig 2 C

3 These C units were *afferent* with partially overlapping receptive fields

II Identification of sympathetic units in the C responses

The collision technique which relies on the fact that impulses which travel in opposite directions in a nerve fibre extinguish each other if they collide was used by Douglas and Ritchie (1957) to study types of active fibres in whole nerve trunks and by Iggo (1958) to identify individual C fibres in multifibre strands In our recordings on alert human subjects collision effects could be demonstrated in sympathetic fibres by eliciting antidromic impulses with electric shocks in the skin and orthodromic sympathetic activity by different arousal manoeuvres

Limitations The lowest electrical stimulation frequency f at which one random sympathetic impulse always will collide with an antidromic impulse is given so long as adjustments are made for the effect of the refractory period by the equation

$$f = \frac{v}{2d}$$

where d is the distance from the stimulating site to the ganglion and v is the conduction velocity in the fibre (*cf* Iggo 1958) Suppose that the distance from the third finger to the autonomic ganglion was at least 80 cm and that the conduction velocity in the sympathetic postganglionic fibre was about 1 m/s then antidromic impulses at frequencies above 0 6 Hz would always collide with a single sympathetic impulse along the fibre If the distance between the ganglion and the recording site is relatively long for example when recording at the wrist there would be a large possibility that the collisions would occur proximal to the recording site and then extinctions or changes in latency would not appear in the centripetally conducted C responses Thus the possibility to classify antidromic activity of sympathetic fibres in the centripetally conducted C responses will be reduced if the stimulation frequency is high (Fig 6 D) and if recordings are made at a long distance from the ganglion Furthermore the identification of such sympathetic units that discharge repeatedly in reflex responses is favoured in comparison with those that only respond with single discharges (Hallin and Torebjork 1974 b) Finally there might exist sympathetic fibres to the skin which are not spontaneously active and cannot be activated by the reflex stimuli used If such fibres responded to the electrical stimulation they could not be classified

Conclusions Under favourable conditions as in the experiment illustrated in Fig 4—6 several inferences about the sympathetic activity can be made

1 The units y and in Fig 4 and 6 were sympathetic since collisions or latency changes could be demonstrated in association with increased sympathetic activity

2 The conduction velocities in distal segments of these sympathetic fibres were approximately 1 0 and 0 93 m/s

3 These units were active not only in reflex responses to loud noises and heavy breathing but also in reflex responses to electrical stimulation since collision effects were demonstrated in these responses (Fig 5)

4 The units also exhibited a sparse spontaneous firing since irregularly occurring discharges in between the reflex bursts provoked occasional collisions or latency

changes of centripetally conducted responses in *y* or *z* on low frequency stimulation

5 Aside from low amplitude massdischarges not prominent in the recording the reflex responses were derived from activity in only three distinct units one large and two small the latter ones were identical with units *y* and *z* in the C response This was infered from the observation that only discharges in the large unit were recorded in sympathetic reflex responses whereas the reflex discharges of other units were totally extinguished by collisions proximal to the recording site on high frequency antidromic activation of *y* and *z* (Fig 6 D)

This information has been applied in studies of single unit sympathetic activity in human skin nerves (Hallin and Torebjork 1974 b)

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References

Bishop G H The action potentials at normal and depressed regions of non myelinated fibers with special reference to monophasic lead *J cell comp Physiol* 1934 *5* 151—169

Delius W K E Hagbarth A Hongell and B G Wallin Manoeuvres affecting sympathetic outflow in human skin nerves *Acta physiol scand* 1972 *84* 177—186

Douglas W W and J M Ritchie A technique for recording functional activity in specific groups of medullated and non medullated fibres in whole nerve trunks *J Physiol* (Lond.) 1957 *138* 19—30

Franz D N and A Iggo Conduction failure in myelinated and non myelinated axons at low temperatures *J Physiol* (Lond) 1968 *199* 319—345

Gasser H S Changes in nerve potentials produced by rapidly repeated stimuli and their relation to the responsiveness of nerve to stimulation *Amer J Physiol* 1935 *111* 35—50

Grundfest H and H S Gasser, Properties of mammalian nerve fibers of slowest conduction *Amer J Physiol* 1938 *123* 307—318

Hagbarth K E A Hongell R G Hallin and H E Torebjork Afferent impulses in median nerve fascicles evoked by tactile stimuli of the human hand *Brain Res* 1970 *24* 423—442

Hagbarth K E R G Hallin A Hongell H E Torebjork and B G Wallin General characteristics of sympathetic activity in human skin nerves *Acta physiol scand* 1972 *84* 164—176

Hallin R G Blocking effects of a topical anesthetic composition containing Ketocaine® on cutaneous C receptor responses in alert man *Acta Anaesthesiol Scand* 1974 In press

Hallin R G and H E Torebjork C fibre components in electrically evoked compound potentials recorded from human median nerve fascicles in situ *Acta Soc Med upsalien* 1970 a *75* 77—80

Hallin R G and H E Torebjork Afferent and efferent C units recorded from human skin nerves in situ *Acta Soc Med upsalien* 1970 b *75* 277—281

Hallin R G and H E Torebjork Electrically induced A and C fibre responses in intact human skin nerves *Exp Brain Res* 1973 *16* 309—320

Hallin R G and H E Torebjork Activity in unmyelinated nerve fibres in man *Advanc Neurol* 1974 a *4* 19—27

Hallin R G and H E Torebjork Single unit sympathetic activity in human skin nerves during rest and various manoeuvres *Acta physiol scand* 1974 b *92* 303—317

van Hees J and J M Gybels Pain related to single afferent C fibers from human skin *Brain Res* 1972 *48* 397—400

Hensel H and K K A Boman Afferent impulses in cutaneous sensory nerves in human subjects *J Neurophysiol* 1960 *23* 564—578

Hering E Beitrage zur allgemeinen Nerven und Muskelphysiologie Über positive Nachschwankung des Nervenstromes nach elektrischer Reizung Wien Kaiserliche Akad der Wissenschaften Sitzungsb Matematisch naturwiss Abt 3 1884 *89*—*90* 137—158

IGGO A The electrophysiological identification of single nerve fibres with particular reference to the slowest-conducting vagal afferent fibres in the cat *J Physiol* (Lond) 1958 *142* 110—126

MATTHEWS B H C The response of a muscle spindle during active contraction of a muscle *J Physiol* (Lond) 1931 *72* 153—174

RITCHIE J M and R W STRAUB The hyperpolarization which follows activity in mammalian non medullated fibres *J Physiol* (Lond) 1957 *136* 80—97

TOREBJORK H E Afferent C units responding to mechanical thermal and chemical stimuli in human non glabrous skin *Acta physiol scand* 1974 *92* 374—390

TOREBJORK H E and R G HALLIN C-fibre units recorded from human sensory nerve fascicles in situ *Acta Soc Med upsalien* 1970 *75* 81—84

TOREBJORK H E and R G HALLIN Perceptual changes accompanying controlled preferential blocking of A and C fibre responses in intact human skin nerves *Exp Brain Res* 1973 *16* 321—332

TOREBJORK H E and R G HALLIN Identification of afferent C units in intact human skin nerves *Brain Res* 1974 a *67* 387—403

TOREBJORK H E and R. G HALLIN Responses in human A and C fibres to repeated electrical intradermal stimulation *J Neurol Neurosurg Psychiat* 1974 b *37* 653—664

VALLBO A B and K E HAGBARTH Activity from skin mechanoreceptors recorded percutaneously in awake human subjects *Exp Neurol* 1968 *21* 270—289

Acta physiol scand 1974 92 332—340

From the Department of Clinical Physiology and the Burn Center University Hospital Uppsala the Department of Clinical Physiology Regionssjukhuset, Linkoping Sweden and the Department of Physiology University of Odense Denmark

Effect of 2, 3-Diphosphoglycerate on the Oxygen Affinity and on the Proton- and Carbamino-Linked Oxygen Affinity of Hemoglobin in Human Whole Blood

By

G ARTURSON L. GARBY B WRANNE and B ZAAR

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Abstract

ARTURSON G L. GARBY B WRANNE and B ZAAR. *Effect of 2,3 diphosphoglycerate on the oxygen affinity and on the proton and carbamino-linked oxygen affinity of hemoglobin in human whole blood* Acta physiol scand 1974 92 332—340

Oxygen binding curves were determined at two plasma pH values in the range 7.0—7.5 each at two pCO_2 values of 22 and 77 mm Hg respectively on human whole blood where the red cell 2,3-diphosphoglycerate (DPG) concentration had been decreased to about 1 mmol/l of packed cells and increased to about 9 mmol/l of packed cells Numerical values are given for the oxygen saturation-dependant ligand interaction coefficients $\Delta \log pO_2/\Delta pH$ $\Delta \log pO_2/\Delta \log pCO_2$ and $\Delta \log pO_2/\Delta \log DPG$ and their dependance on pH pCO_2 and DPG respectively

The molecular mechanisms by which the 4 main physiological ligands interact with hemoglobin have recently been elucidated to a considerable extent (see for example Perutz 1970 and Kilmartin and Rossi Bernardi 1973) The thermodynamical relationships describing these interactions have been reviewed recently (Siggaard Andersen *et al* 1972 b) and numerical values of several of the interaction coefficients valid for human whole blood and concentrated hemoglobin solutions, have now been estimated (Siggaard Andersen 1971 Duhm 1971 Siggaaard Andersen and Salling 1972 Siggaard Andersen *et al* 1972 a Garby Robert and Zaar 1972 and Wranne Woodson and Detter 1972) However there is still a lack of data in some important areas In particular the dependance of the ligand interaction coefficients on the oxygen saturation level has been determined only at the normal range of red cell 2,3-diphosphoglycerate concentration (DPG) (Garby Robert and Zaar 1972) In the present paper we present the results of experiments carried out

on whole blood in which the red cell content of DPG was varied in order to study the effect of this compound on the oxygen affinity of the blood and in particular on the proton and carbamino linked oxygen affinity

Parameters of ligand interaction in whole blood are of interest primarily for studies on the respiratory function of the blood and the main contribution of this paper is to present numerical values of such parameters Since however such data can also be interpreted in terms of ligand binding through the reciprocal relations of Wyman (1948 1964 see also Garby *et al* 1972) they are of importance also in attempts to describe the molecular events that take place during the successive oxygenation of the hemoglobin molecule Some of the implications of the present kork on this particular aspect of hemoglobin ligand interaction will be discussed

Methods

Blood from healthy non smoking individuals was used Increased red cell DPG content was achieved as described by Deuticke Duhm and Dierkesmann (1971) After centrifugation of the heparinized (0 4 mg/ml) blood plasma was taken off and the cells washed 3 times with an excess of Locke s solution The cells were thereafter incubated at 37 C for 1 h in 10 volumes of saline medium containing inosine (10 mmol/l) pyruvate (10 mmol/l) and inorganic phosphate (25 mmol/l) After rapid cooling the cells were washed at 0 C 3 times in a 10 fold volume of Locke s solution Red cell DPG depletion was achieved by ten days storage of whole blood in acid citrate dextrose solution (NIH formula B) at 4 C The cells were washed 3 times in a 10-fold volume of Locke's solution and resuspended in heparinized plasma obtained from the same individual At both DPG levels 2 5 N NaOH or HCl was added to the plasma of 4 aliquots of blood in amounts yielding 4 blood samples with pH values of about 7 2 and 7 5 at pCO levels of 22 and 77 mm Hg respectively

Oxygen dissociation curves were determined in the Radiometer DCA 1 Instrument (Radiometer Copenhagen Denmark) as described previously (Duvelleroy *et al* 1970 cp also Garb *et al* 1972) DPG concentrations were determined immediately before and after the run (Eriksson and de Verdier 1972) Carbon monoxide hemoglobin (COHb) was determined by the method of Dahlstrom (1960) as modified by Gassmann and Wranne (1967) and spectrophotometrically in the IL 182 Instrument (Instrumentation Laboratories Lexington Mass U S A) according to the manufacturers manual Methemoglobin determination was made according to Evelyn and Malloy (1938) Hemoglobin concentration was determined as cyanmethemoglobin and hematocrit by the microhematocrit technique

4 oxygen binding curves were performed on each of 6 different bloods with a low DPG concentration and on each of 6 different bloods with a high DPG concentration 2 of the 4 curves were run at a high plasma pH value (7 3—7 5) and 2 at a low plasma pH value (7 0—7 3) and in each of these 2 sets of pH values one curve was run at a pCO_2 of 22 mm Hg and another at a pCO_2 of 77 mm Hg

The hemoglobin concentration and the hematocrit value measured immediately before and after each run were the same in both kinds of blood with a mean value of 14 5 g/l and 44 respectively The COHb concentration measured also immediately before and after each run was 2 8 % in the low DPG blood and 2 0 % in the high DPG blood with no single value higher than 4 0 The red cell DPG concentration was 1 75 mmol/l of packed cells in the samples run at a pCO_2 of 22 mm Hg (n = 12 s e m = 0 10) and 1 27 mmol/l of packed cells in the samples run at a pCO_2 of 77 mm Hg (n = 11 s e m = 0 18) in the low DPG blood and 9 32 (n = 11 s e m = 0 19) and 9 43 (n = 8 S E = 0 20) respectively in the high DPG blood There were no significant differences in either of the 4 blood parameters between the values obtained before and after the oxygen binding curve measurements The methemoglobin concentration measured before and after the runs was less than 2

For each oxygen saturation level of 10 20 30 40 50 60 70 80 90 95 and 97 5 respectively the $\log pO_2$ values were plotted against the plasma pH values as measured directly in the apparatus Straight lines were fitted to the data obtained on bloods with a high DPG value while curves were fitted by eye through the data obtained with the low DPG blood samples

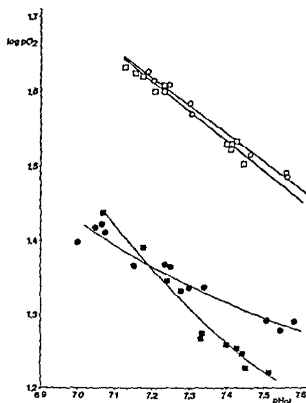


Fig 1 The relation between $\log pO_2$ and pH (plasma) at 50 % oxygen saturation O DPG = 9 4 mmol/l and pCO_2 = 77 mm Hg □ DPG = 9 3 mmol/l and pCO_2 = 27 mm Hg ● DPG = 1 27 mmol/l and pCO_2 = 77 mm Hg ■ DPG = 1 75 mmol/l and pCO_2 = 27 mm Hg

Results

The results at 50 % oxygen saturation are shown in Fig 1 these results are representative for the other saturation levels both with respect to the scatter of the data and the curvilinearity of the relation at low DPG concentrations

The results at 50 % saturation and a plasma pH of 7 40 are shown in Fig 2 as a function of the red cell DPG concentration and in relation to previously published data *

The quotient $\Delta \log pO_2/\Delta pH_{pl}$ at a plasma pH of 7 40 is shown in Fig 3 as a function of the oxygen saturation

The intracellular pH pH_c was calculated in each case from the plasma pH, the red cell DPG concentration and the oxygen saturation according to the relations given by Duhm (1971) The resulting values of $\Delta pH_{pl}/\Delta pH_c$ were then used to obtain $\Delta \log pO_2/\Delta pH_c$ at each saturation level from the smoothed curves in Fig 3 The results are shown in Fig 4

* The data points referring to the previous publication from our laboratory (Garby et al 1972) have been recalculated from the original data. In the previous publication the data at high and low pCO_2 were assumed to have identical values of the red cell DPG i e the same mean value of 4 25 mmol/l packed red cells A closer inspection of the data revealed however that the mean red cell DPG concentration in the runs with a high pCO_2 was somewhat higher 4 48 mmol/l than that in the samples run at a low pCO_2 4 01 mmol/l

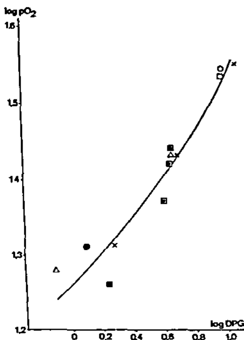


Fig 2

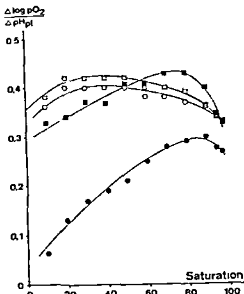


Fig 3

Fig 2 The relation of $\log pO_2$ at 50 % oxygen saturation to the red cell DPG concentration (mmol/l) at a plasma pH of 7 40 and various pCO_2 values O ● and □ ■ present data at pCO_2 77 and 27 mm Hg respectively ⊡ data from Garby Robert and Zaar (1972) at pCO_2 values of 22 34 and 83 mm Hg × data from Duhm (1971) at $pCO_2 = 40$ mm Hg △ data from Wranne *et al* (1972) at $pCO_2 = 48$ mm Hg The curve is drawn for a pCO_2 value of 40 mm Hg

Fig 3 The relation between $\Delta \log pO_2/\Delta pH_{pl}$ at $pH_{pl} = 7\,40$ and the oxygen saturation O DPG = 9 4 mmol/l and $pCO_2 = 77$ mm Hg □ DPG = 9.3 mmol/l and $pCO_2 = 22$ mm Hg ● DPG = 1 27 mmol/l and $pCO_2 = 77$ mm Hg ■ DPG = 1 75 mmol/l and $pCO_2 = 27$ mm Hg

The integrated values of Fig 4 *i e* the normalized areas under each curve are shown in Fig 5 in relation to previously published data

The values of $\log pO_2$ at $pH_e = 7\,20$ from the present experiments as well as from previously published figures are shown in Fig 6 as a function of logDPG for several levels of oxygen saturation

Fig 7 shows the carbamino-linked oxygen affinity *i e* $\Delta \log pO_2/\Delta \log pCO_2$ determined for the pCO_2 range of 22—77 mm Hg at different red cell DPG concentrations The data points are calculated directly from Fig 6

The ratio $\Delta \log pO_2/\Delta \log DPG$ determined for the DPG range of 1 5—9 3 mmol/l packed cells at different pCO_2 values is shown in Fig 8

Discussion

The oxygen affinity of whole blood depends primarily on the chemical potentials of the effector ligand protons carbon dioxide and DPG The effect of other

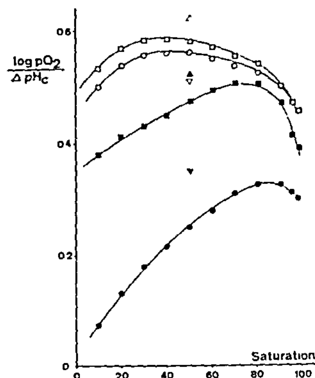


Fig 4 The relation between $\Delta \log pO_2/\Delta pH_c \sim 7\,20$ and the oxygen saturation □ O and ■ ● present data with symbols as in Fig 3 △ and ▽ data from Siggaard Andersen *et al* (19 ?), at DPG ≈ 2 9 and 0 mmol/l, respectively and $pCO_2 = 0$ mm Hg ▲ and ▼ data from Siggaard Andersen *et al* (19?2) at DPG 3 4 and 0 mmol/l respectively and $pCO_2 = 32$ mm Hg

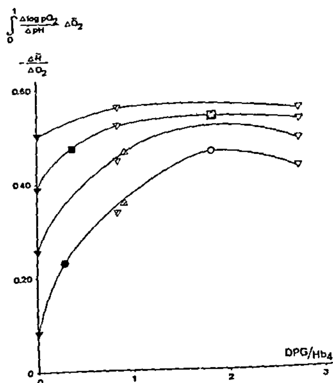


Fig 5 Effect of DPG concentration expressed as the molar ratio of DPG and hemoglobin tetramer on the oxygenlinked proton binding $-\Delta\bar{H}/\Delta\bar{O}$ (▼ ▽ ▽ data from S ?gard Andersen 1971) and on th integrated proton linked oxygen affinity (△ O ■ ● present data ∠ data from Garby *et al* (197?) The 4 curves drawn by eye correspond to the fo owing values for pCO_2 0 ?? 34 and 80 mm Hg as read from the top

Fig 6 The relation between log pO_2 at $pH_c = 7.20$ and log DPG at several levels of oxygen saturation ● ○ present data at $pCO_2 = 77$ mm Hg ■ □ present data at pCO = 2? mm Hg ▲ △ data from Garby *et al* (1972) at 83 and 22 mm Hg respectively × data from Duhm (1971) at pCO = 40 mm Hg

ligands e g carbon monoxide inorganic phosphate and organic phosphates other than DPG is generally small and has been neglected in the present system The observed carbon monoxide hemoglobin concentrations (2—2 8 %) are somewhat larger than are generally found (1—2 %) in non smokers No explanation can be offered for this finding According to Roughton (1964) a difference in the COHb concentration of 1 % will change the oxygen tension at half saturation by about 0 28 mm Hg Such an effect would not be detected by the technique used in the present study The erythrocyte content of inorganic phosphate and of organic phosphates other than DPG after biochemical manipulations of the type used here has been studied by Duhm (1971) The molar concentration of such compounds

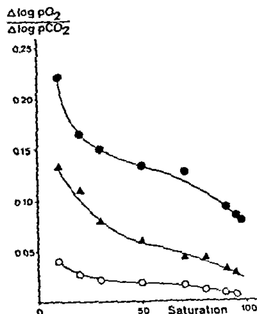


Fig 7

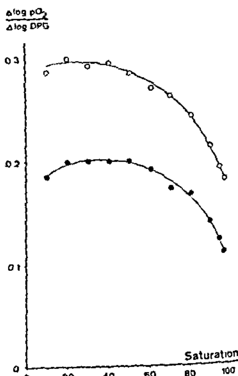


Fig 8

Fig 7 The relation between $\Delta\log pO_2/\Delta\log pCO_2$ at $pH_e = 7.20$ and oxygen saturation for the pCO_2 range of 22—77 mm Hg at different red cell DPG concentrations The data points are obtained directly from Fig 6 ● DPG = 1 2 mmol/l ▲ DPG ~ 5 0 mmol/l ○ DPG = 9 0 mmol/l

Fig 8 The relation between $\Delta\log pO_2/\Delta\log DPG$ and the oxygen saturation for the DPG range of 1 5—9 3 mmol/l ○ data at pCO_2 = 22 mm Hg ● data at pCO_2 = 77 mm Hg The cross refers to data of Duhm (1971) at a pCO_2 of 40 mm Hg

and the variations encountered were found to be insignificant with respect to their effect on the hemoglobin oxygen affinity The effect on the Donnan distribution of diffusible ions is not negligible however but was accounted for in the present study by using the distributions actually found by Duhm (1971) Differences in plasma pH between the experiments and changes in pH during the experiments have an influence on the erythrocyte volume Data obtained by Funder and Wieth (1966 a and b) have shown however that during experiments of the type performed here a change in pH of 0 1 unit obtained through addition of acid or base or through changes in pCO_2 would change the hematocrit by only about 0 5 % absolute value

The present data confirm and extend the previous observations (Garby *et al* 1972) concerning the non linearity with respect to oxygen saturation of both the proton and the carbamino-linked oxygen affinity Wherever comparison is possible i e at 50 % oxygen saturation and for the integrated oxygen saturation the present

data are in good agreement with the results of Siggaard Andersen and collaborators (see introduction) and with the data of Duhm (1971)

An explicit model for allosteric transitions and ligand binding of the hemoglobin molecule is still lacking The present data can therefore be rigorously interpreted only at the level of the ligand interaction coefficients *i e* the relations between the activities of two ligands at fixed values of all other ligand or the relations between the binding of two ligands at fixed values of all other ligands Such data now available for physiologically relevant ranges of the activities (concentrations) of all the ligands are however of fundamental importance in applied research of the respiratory function of the blood

Our previous interpretation of the non linearity of the CO linked oxygen affinity (Garby *et al* 1972) *i e* that most of the carbamino binding takes place to the α chains in the presence of DPG is supported by the recent results of Gibson (1973) that the α chains have a higher affinity to oxygen than the β chains It is also supported by the recent data of Kilmartin Fogg Luzzana and Rossi Bernardi (1973) who showed that in the presence of DPG the effect of CO_2 on the oxygen affinity of tetramers carbamylated in the β chain termini is larger than that of tetramers carbamylated in the α chain termini On the other hand the present data indicate that the same interpretation would be valid also in the absence of DPG This result is not immediately reconcilable with the data of Kilmartin *et al* (1973) who found that in the absence of DPG the CO effect on the oxygen affinity was larger in the case of hemoglobin carbamylated in the α chain termini than in the case of hemoglobin carbamylated in the β chain termini However since carbamylation at the α chain termini caused a large change in the oxygen affinity also in the absence of DPG and CO these results must be interpreted with caution

Applying the reciprocal relation of Wyman (1948 1964) to obtain estimates of the oxygen linked carbamino binding from the observed values of $\Delta \log p\mathrm{O}$ / $\Delta \log p\mathrm{CO}$ gives a value of 0 08 mol/mol for the average oxygen linked carbamate of hemoglobin A under normal intracellular conditions (Fig 7) This estimate is in excellent agreement with the figure obtained by Bauer and Schroder (1972) using a more direct method of estimation The present data (*cf* also Garby *et al* 1972) show however that the oxygen linked carbamate formation i strongly dependent on the oxygen saturation level Therefore any estimate of the relative contribution of oxygen linked carbamate formation to the total CO tran port depends strongly on the degree of desaturation considered For the situ ation considered by Bauer and Schroder (1972) *i e* desaturation of normal arterial blood at $p\mathrm{CO} = 40$ mm Hg to a saturation of 75 % and a $p\mathrm{CO}$ of 47 mm Hg the relative contribution of the carbamate formation to the total CO transport work out to be only 1/3—1/2 of that estimated by Bauer and Schroder (1972) or only about 3—5 %

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References

BAUER C and E SCHRODER Carbamino compounds of haemoglobin in human adult and foetal blood *J Physiol* (Lond) 1972 *227* 457—471

DAHLSTROM H A method for determination of carbon monoxide in gas phase applied for determination of the carbon monoxide capacity of blood *Scand J clin Lab Invest* 1960 *12* 396—402

DEUTICKE B J DUHM and R DIERKESMANN Maximal elevation of 2 3-diphosphoglycerate concentrations in human erythrocytes Influence on glycolytic metabolism and intracellular pH *Pflugers Arch ges Physiol* 1971 *326* 15—34

DUHM J Effects of 2 3 diphosphoglycerate and other organic phosphate compounds on oxygen affinity and intracellular pH of human erythrocytes *Pflugers Arch ges Physiol* 1971 *326* 341—356

DUVELLEROY M A R G BUCKLES R ROSENKAIMER C TUNG and M B LAVER An oxyhemoglobin dissociation analyzer *J appl Physiol* 1970 *28* 227—233

ERICSON A and C H DE VERDIER Modified method for the determination of 2 3-diphosphoglycerate in erythrocytes *Scand J clin Lab Invest* 1972 *29* 85—90

EVELYN K A and H T MALLOY Microdetermination of oxyhemoglobin methemoglobin and sulfhemoglobin in a single blood sample *J biol Chem* 1938 *126* 655—662

FUNDER J and J O WIETH Cloride and Hydrogen Ion Distribution between Human Red Cells and Plasma *Acta physiol scand* 1966 *68* 234—245

FUNDER J and J O WIETH Determination of Sodium Potassium and Water in Human Red Blood Cells *Scand J clin Lab Invest* 1966 *18* 151—166

GARBY L M ROBERT and B ZAAR Proton and carbamino-linked oxygen affinity of normal human blood *Acta physiol scand* 1972 *84* 482—492

GASSMANN H B and L WRANNE Microdetermination of carbon monoxide in blood *Acta Soc Med upsalien* 1967 *72* 277—282

GIBSON Q H The contribution of the α and β chains to the kinetics of oxygen binding to and dissociation from hemoglobin *Proc Nat Acad Sci* (Wash) 1973 *70* 1—4

KILMARTIN J V J FOGG M LUZZANO and L ROSSI BERNARDI Role of the α amino groups of the α and β chains of human hemoglobin in oxygen linked binding of carbon dioxide *J biol Chem* 1973 *248* 7039—7043

KILMARTIN J V and L ROSSI BERNARDI Interaction of hemoglobin with hydrogen ions carbon dioxide and organic phosphates *Physiol Rev* 1973 *53* 836—890

PERUTZ M F Stereochemistry of cooperative effects in hemoglobin *Nature* (Lond) 1970 *228* 726—739

ROUGHTON F J W Transport of oxygen and carbon dioxide In *Handbook of Physiol* section 3 Respiration Vol I ed Fenn W O and H Rahn 1964 767—825 Amer Physiol Soc Washington DC

SIGGAARD ANDERSEN O Oxygen linked hydrogen ion binding of human hemoglobin Effects of carbon dioxide and 2 3 diphosphoglycerate I Studies on erythrolysate *Scand J clin Lab Invest* 1971 *27* 351—360

SIGGAARD ANDERSEN O and N SALLING Oxygen linked hydrogen ion binding of human hemoglobin Effects of carbon dioxide and 2 3 diphosphoglycerate II Studies on whole blood *Scand J clin Lab Invest* 1971 *27* 361—366

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SIGGAARD ANDERSEN O M RORTH B NORGAARD PEDERSEN O SPARRE ANDERSEN and E JOHANSEN Oxygen linked dioxide and 2 3 diphosphoglycerate IV Thermodynamical relationship between the variables *Scand J clin Lab Invest* 1972 b *29* 303—320

WRANNE B R D WOODSON and J C DETTER Bohr effect interaction between H CO2 and 2 3 DPG in fresh and stored blood *J appl Physiol* 1972 *32* 749—754

WYMAN J Heme proteins *Advanc Protein Chem* 1948 *4* 407—531

WYMAN J Linked functions and reciprocal effects in hemoglobin A second look *Advanc Protein Chem* 1964 *19* 223—286

Acta physiol scand 1974 92 341—350

From the Nobel Institute for Neurophysiology Karolinska Institutet and the Department of Automatic Control Royal Institute of Technology Stockholm Sweden

Transient and Steady State Effects of CO on Mechanisms Determining Rate and Depth of Breathing

By

G W Bradley[1] C von Euler Irja Marttila and B Roos

Received 17 April 1974

Abstract

Bradley G W C von Euler I Marttila and B Roos *Transient and steady state effects of CO_2 on mechanisms determining rate and depth of breathing* Acta physiol scand 1974 92 341—350

Breath by breath analysis of tidal volume V_T inspiratory duration T_I and expiratory duration T_E was performed on cats under light pentobarbitone anesthesia or after decerebration before and after vagotomy In response to step changes in inspired CO V_T changes earlier and more rapidly than the corresponding changes in the timing The V_T—T_I points may thus form a hysteresis loop embracing the volume threshold curve of Clark and Euler (1972) The transient responses were present also after peripheral chemodenervation and after bilateral vagotomy In contrast to earlier papers vagotomized cats under pentobarbitone anesthesia often showed some decrease of T_I with increased steady state CO_2 levels In decerebrate vagotomized cats T_I was more independent of variations in steady state PCO levels but after administration of pentobarbitone T_I showed some steady state shortening of T_I with increased CO_2 It is concluded that the controller of depth and rate of breathing contains at least 2 functional components which depend on PCO in such a way that they exert opposite effects on T_I which cancel out to a great extent and that this relative match can be disturbed by pentobarbitone It is postulated that the transient increase in mismatch is due to the fact that the 2 (or more) CO effects have slightly different dynamic features

In previous work from this laboratory (Euler Herrero and Wexler 1970 Clark and Euler 1972 Knox 1973 Euler *et al* 1973) some problems concerning the control of rate and depth of breathing have been analysed both in the presence and absence of afferent feed back from the lungs In the absence of volume information from the lungs it has been reported that the duration of the breath cycle is not related to either tidal volume or to the level of chemical drive giving a relatively constant respiratory rate in spite of large changes in chemical drive (Scott 1908 Cohen 1964 Nesland *et al* 1966 Richardson and Widdicombe 1969 Euler *et al* 1970 Clark and Euler 1972 Grunstein Younes and Milic Emili 1973) This is in good agree

[1] Present address Department for Respiratory Investigation Glasgow Royal Infirmary Glasgow Scotland

References

BAUER C and F SCHRÖDER Carbamino compounds of haemoglobin in human adult and foetal blood *J Physiol* (Lond) 1972 *227* 457—471

DAHLSTRÖM H A method for determination of carbon monoxide in gas phase applied for determination of the carbon monoxide capacity of blood *Scand J clin Lab Invest* 1960 *12* 396—402

DEUTICKE B J DUHM and R DIERKESMANN Maximal elevation of 2 3-diphosphoglycerate concentrations in human erythrocytes Influence on glycolytic metabolism and intracellular pH *Pflugers Arch ges Physiol* 1971 *326* 15—34

DUHM J Effects of 2 3 diphosphoglycerate and other organic phosphate compounds on oxygen affinity and intracellular pH of human erythrocytes *Pflugers Arch ges Physiol* 1971 *326* 341—356

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SIGGAARD-ANDERSEN O M RØRTH B NØRGAARD PEDERSEN O SPARRE ANDERSEN and E JOHANSEN Oxygen linked dioxide and 2 3-diphosphoglycerate IV Thermodynamical relationship between the variables *Scand J clin Lab Invest* 1972 b *29* 303—320

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WYMAN J Heme proteins *Advanc Protein Chem* 1948 *4* 407—531

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Routine surgical preparation consisted of tracheal cannulation catheterization of a femoral artery and vein and preparation of the vagus nerves for subsequent section In 3 cats the carotid sinus nerves were exposed bilaterally and were cut later in the experiment The identity of the nerves was checked by recording the blood pressure response to carotid artery obstruction before and after cutting

The efferent activity in the central stump of the phrenic C5 root was recorded and integrated by means of a rectifier and R C network The integrated phrenic activity was used to produce a square wave signal which changed phase at the beginning and end of inspiration The voltage level and thereby the time at which the phase changes occurred were set so that a minimal delay was introduced yet avoiding spurious signals produced by noise on the baseline The square wave signal obtained was recorded on a four channel magnetic tape (F M recorder Precision Instrument) and subsequently used for measurement of inspiratory duration (T_I) and expiratory duration (T_E) An analogue voltage of T_I was also available and recorded on a chart recorder

Airflow was recorded using a Fleish pneumotachograph (size 00) and differential pressure transducer Inspiratory airflow was integrated to give tidal volume (V_T) using an operational amplifier with feed back capacitance Expiratory flow was used to reset and maintain the integrator at zero volts until the onset of the next inspiration The V_T signal was recorded on magnetic tape and on the chart recorder It was also put into a peak follower circuit the output of which was recorded on the Y axis of a storage oscilloscope (Tektronix R 564B) The sweep was triggered at the beginning of inspiration and the beam brightened momentarily at the end of inspiration using the square wave signal from the phrenic discharge A plot of V_T (Y axis) against T_I (X axis) was thus displayed on the oscilloscope screen and was used for on line assessment of the results

In four experiments the cats were placed in a body plethysmograph connected to a Krogh spirometer an electrical output from which was recorded on the chart recorder The purpose of these experiments was to assess changes in functional residual capacity (FRC) This was achieved indirectly by measuring the expiratory reserve volume ERV (Euler and Fritts 1963)

A large bag ($\simeq 50$ l) was connected to the animal via a system circulating the gas rapidly and by quickly switching to a bag containing a different gas mixture almost stepwise changes in the inspired gas could be produced The CO mixtures were applied until a new steady state had been reached or longer *i e* for 3 to 10 min and then removed The end tidal CO_2 concentration was measured using an infrared CO_2 analyser (Beckman LBI) This was uncoupled from the animal when data was being recorded to prevent any influence of the sampling rate on the airflow signal

The data recorded on tape was subsequently analysed off line using a PDP7 digital computer T_I and T_E were measured by counting clock pulses during the inspiratory and expiratory phase of the square wave signal triggered from the phrenic activity and the analogue voltage of V_T was converted into digital form The computer calculated the duration of the breath the rate of breathing and the total ventilation a plot of any combination of these could be displayed on a TV screen or plotted out using an XY recorder Data resulting from spurious pulses and false triggering could be deleted by a special programme routine Obvious sighs or augmented breaths were also deleted on this programme

For computational reasons it was easier to use tidal volume rather than the volume reached at the time when the phrenic activity was switched off (*i e* V_{Teq} of Clark and Euler 1972) although the latter entity might be somewhat more closely related to the inhibitory discharge from the stretch receptors of the lungs implicated in the inspiratory termination Clark and Euler (1972) found a difference between the two which increased with increased tidal volumes and amounted to a maximum of 15% error at the largest inflation used This would tend to make the V_T—T_I curve slightly more curvilinear than the corresponding measured V_{Teq}—T_I curve

Results

Intact vagus nerves

In response to a sudden change in composition of the inspired gas from air to a CO air mixture an increase in tidal volume occurred before an increase in respiratory rate and the shortening in inspiratory and expiratory durations became evident (Loeschcke *et al* 1963) In fact it was commonly observed that the inspiratory duration initially showed a slight prolongation. This only lasted for a few breaths but the time course of the subsequent changes demonstrated a clear phase shift in

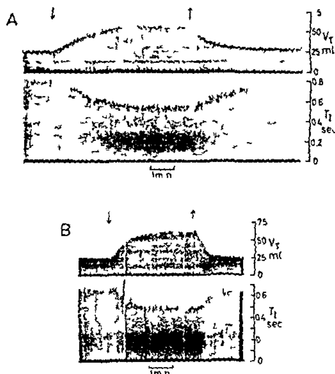


Fig 1 The time courses of tidal volume V_T (upper trace) and inspiratory duration T_I (lower trace) in response to step changes in inhaled CO_2 concentration A From a cat under light pentobarbitone anesthesia. Between arrows a CO_2 enriched gas mixture was given F_ICO_2 rose from 4 0 % to 7 6 % and returned when breathing air again to 4 3 % B From a decerebrate cat with the changes in F_ACO_2 from 3 4 % to 5 3 % and back to 3 3 %

the transient response of tidal volume V_T and inspiratory duration T_I with a tendency for V_T to lead over T_I and to reach its new steady state level while T_I was still slowly decreasing Similarly removal of the CO_2 mixture and return to air produced earlier and more rapid changes in V_T than T_I This is illustrated in Fig 1 A plot of V_T against T_I during these manoeuvres consequently showed hysteresis as shown in Fig 2

With lightly anesthetized and decerebrate preparations both V_T and T_I had reached a new steady state by at the most 5 minutes after application of the gas mixture containing CO_2 Small changes that sometimes occurred after this time were difficult to distinguish from possible effect of spontaneous changes in the central excitatory state of the animal

This type of transient response to step changes in inspired CO_2 was seen both in the anesthetized and decerebrate animals but the amount of hysteresis varied considerably Only in seven out of 61 trials could a hysteresis effect not be detected with certainty

Fig 2 B shows that the relationship between expiratory and inspiratory duration (T_E *vs* T_I) was not significantly subjected to similar transient effects

Peripheral chemoreceptor influence

This phase difference between the time course of the V_T and T_I responses to step changes in inhaled CO_2 was not changed by substituting oxygen for air in the bag

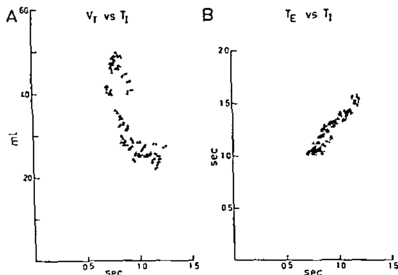


Fig 2 From a cat under light pentobarbitone anesthesia A The transient effects on the V_T—T_I relationships showing a typical hysteresis loop in response to step changes in the inhaled CO_2 concentration B The corresponding T_E—T_I relationship Crosses denote the response to a rapid increase in F_ICO_2 from 3 8 % (breathing air) to 8 0 % and the triangles denote the response back to breathing air with F_ICO_2 of 3 8 %

containing the CO_2 mixture despite consequent depression of the input from the peripheral chemoreceptors Similarly section of the carotid sinus nerves in experiments using air and CO_2 air mixtures did not abolish the hysteresis These findings indicate that the afferent input from the peripheral chemoreceptors was not a determining factor in producing the phase differences in the transient CO_2 responses (*cf* Berger Krasney and Dutton 1973 Pearson and Cunningham 1973 Cunningham *et al* 1974)

Effect of vagotomy

Transient responses The effect of changing the inhaled CO_2 on the discharge of the pulmonary receptors (Mustafa and Purves 1972 Schoener and Frankel 1972) might offer an explanation for the transient response described above However similar transients were usually encountered when the experiments were repeated after vagotomy (Fig 3) Marked hysteresis loops after vagotomy were seen in 21 out of 37 trials and only in two cats one decerebrate and one anesthetized with pentobarbitone could hysteresis not be demonstrated in any of the experimental trials After vagotomy it was commonly found that the transient response to a step increase in CO_2 started with a prolongation of T_I followed soon by an increase in tidal volume and a subsequent shortening of T_I to its previous eupnoeic value or even shorter

Steady state responses In earlier papers it has been repeatedly reported that after vagotomy the respiratory rate changes relatively little as tidal volume is increased

hysteresis was recorded also after vagotomy and no obvious influence from chest wall expansions was observed Nevertheless, this possibility was brought to a further test in three animals using a body box to detect changes in FRC We were unable to detect any significant effects of moderate changes in the inspired CO_2 concentration (0—7 %) on FRC either transiently or when steady states had been reached Thus changes in FRC could be ruled out as the cause of the hysteresis effects

Discussion

In full agreement with the results reported by Pearson and Cunningham (1973) for man we have obtained transient effects on the volume—time relationship by changing the inspired CO_2 concentration which are characterized by a lag in the response of T_I relative to V_T, with a hysteresis loop around the steady state V_T—T_I curve No major transient changes in the T_I—T_E relationship were found The transient effects obtained suggest that the mechanisms which determine the V_T—T_I relationship comprise at least two CO_2 dependent components with different dynamic features The present results thus require some modification of or an addition to the basic model of Clark and Euler (1972) for the control of tidal volume and cycle duration This model was derived from nearly steady state responses obtained with relatively slowly changes in the concentrations of inhaled CO_2 From theirs and earlier work (*cf* Euler *et al* 1970) it appeared that the main respiratory parameter which changes with CO_2 is the rate of rise of inspiratory activity and hence the rate of volume expansion during the course of inspiration The results of the present work thus indicate a further CO_2 dependent mechanism which exerts an effect on inspiratory timing in a direction opposite to that exerted through the change in rate of increase in inspiratory activity This conclusion is supported by the finding that after vagotomy T_I may still show some but variable dependence on CO_2 an effect which in several cases was seen only after administration of pentobarbitone The relatively small magnitude of a steady state CO_2 effect on T_I after vagotomy emphasized in earlier papers (Cohen 1964 Nesland *et al* 1966 Clark and Euler 1972) suggests that these mutual opposing effects of CO_2 on the timing mechanism are matching each other to a great though variable extent although this matching may be disturbed by pentobarbitone

The mechanism by which CO_2 exerts its influence other than on the rate of increase in inspiratory activity is not well known The results with vagotomy however excludes the possibility that the CO_2 sensitivity of the vagal receptor discharge recently reported by Mustafa and Purves (1972) and by Schoener and Frankel (1972) is alone responsible for the described effects and the experiments under gallamine paralysis indicate that these effects are not exerted secondarily through chest wall proprioceptors but are largely of central origin In this connection it may be pertinent to refer to the paper by Euler *et al* (1973) in which the neural mechanism underlying the time course of the volume threshold curve was elucidated The results showed that the vagal stretch receptor afferents converged

onto neurons in the solitary tract complex which also received an input of rapidly increasing inspiratory excitatory activity of central origin (CIE) providing the required rapidly growing facilitation of the vagal input during inspiration. It was postulated that this combined vagal and CIE activity projects onto the switch neurons which at a certain threshold (Cohen 1971) start to fire and execute the inspiratory off switch function. This suggests the possibility that the off switch threshold is raised as a direct consequence of increased PCO_2, an effect which would tend to lengthen T_I counteracting the shortening effect on T_I exerted by the increased rate of inspiratory activity.

This hypothesis concerning the required second CO_2 dependent mechanism will be further discussed in subsequent papers (Bradley *et al.* 1974 a, b). In the latter of these papers it will be shown also that the transient divergency from the steady state V_T—T_I *responses to* CO_2 *can be satisfactorily explained by merely assuming only* very small differencies in dynamic features of the two CO_2 dependent structures. Evidence showing the presence of different time constants in the transient responses to CO_2 has recently been presented for dogs also after peripheral chemodenervation (Berger *et al.* 1973).

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References

BERGER, A. J., J. A. KRASNEY and R. E. DUTTON. Respiratory recovery from CO_2 breathing in intact and chemodenervated awake dogs. *J. appl. Physiol.* 1973. 35. 35—41.

BRADLEY, G. W., C. VON EULER, I. MARTTILA and B. ROOS. Steady state effects of CO_2 and temperature on the relationship between lung volume and inspiratory duration (Hering—Breuer threshold curve). *Acta physiol. scand.* 1974 a. 92. 351—363.

BRADLEY, G. W., C. VON EULER, I. MARTTILA and B. ROOS. A model of the reflex inhibition of inspiration in the cat. *Kybernetik* 1975 b. *In press.*

CLARK, F. J. and C. VON EULER. On the regulation of depth and rate of breathing. *J. Physiol.* (Lond.) 1972. 222. 267—295.

COHEN, M. I. Respiratory periodicity in the paralyzed, vagotomized cat: hypocapnic polypnea. *Amer. J. Physiol.* 1964. 206. 845—854.

COHEN, M. I. Switching of the respiratory phases and evoked phrenic responses produced by rostral pontine electrical stimulation. *J. Physiol.* (Lond.) 1971. 217. 133—158.

CUNNINGHAM, D. J. C., S. B. PEARSON and W. N. GARDNER. Regulation of respiratory frequency and tidal volume at various ventilations. *Arch. Fisiol.* 1972. 69. Suppl. 433—446.

EULER, C. VON and H. W. FRITTS JR. Quantitative aspects of respiratory reflexes from the lungs and chest walls of cats. *Acta physiol. scand.* 1963. 57. 284—300.

EULER, C. VON, F. HERRERO and I. WEXLER. Control mechanisms determining rate and depth of respiratory movements. *Resp. Physiol.* 1970. 10. 93—108.

EULER, C. VON, J. N. HAYWARD, I. MARTTILA AND R. J. WYMAN. RESPIRATORY NEURONES OF ventrolateral nucleus of the solitary tract of cat: vagal input, spinal connections and morphological identification. *Brain Res.* 1973. 61. 1—?

GRUNSTEIN, M. M., M. YOUNES and J. MILIC-EMILI. Control of tidal volume and respiratory frequency in anesthetized cats. *J. appl. Physiol.* 1973. 35. 463—476.

GUZ, A. and J. G. WIDDICOMBE. Pattern of breathing during hypercapnia before and after vagal blockade in man. Pp. 41—44 in Porter, R. (ed.) *Breathing: Hering-Breuer Centenary Symposium*. Lond. Churchill 1970.

GUZ, A., M. I. M. NOBLE, J. G. WIDDICOMBE, D. TRENCHARD and W. W. MUSHIN. The effect of bilateral block of vagus and glossopharyngeal nerves on the ventilatory response to CO_2 of conscious man. *Resp. Physiol.* 1966. 1. 206—210.

HEY E N B B LLOYD D J C CUNNINGHAM M G M JUKES and D P G BOLTON Effects of various respiratory stimuli on the depth and frequency of breathing in man. *Resp Physiol* 1966 *1* 193—205

KNOX C K Characteristics of inflation and deflation reflexes during expiration in the cat. *J Neurophysiol* 1973 *36* 284—295

LOESCHCKE H H B KATSAROS C ALBERS and C C MICHEL Über den zeitlichen Verlauf von Atemzugvolumen Atem Periodendauer Atemminutenvolumen und endexspiratorischem CO Druck bei Einatmung von Gasgemischen mit erhöhtem CO_2-Druck *Pflugers Arch ges Physiol* 1963 *277* 671—683

MUSTAFA, M E K Y and M J PURVES The effect of CO upon discharge from slowly adapting stretch receptors in the lungs of rabbits *Resp Physiol* 1972 *16* 197—212

NESLAND R. S F PLUM J R. NELSON and H D SIEDLER The graded response to stimulation of medullary respiratory neurons *Exp Neurol* 1966 *14* 57—76

PEARSON S B and D J C CUNNINGHAM Some observations on the relation between ventilation tidal volume and frequency in man in various steady and transient states *Acta Neurobiol Exp* 1973 *33* 177—188

REYNOLDS W J and H T MILHORN JR. Transient ventilatory response to hypoxia with and without controlled alveolar PCO_2 *J appl Physiol* 1973 35 181—196

RICHARDSON P S and J G WIDDICOMBE The role of the vagus nerves in the ventilatory responses to hypercapnia and hypoxia in anaesthetized and unanaesthetized rabbits. *Resp Physiol* 1969 *7* 122—135

SCHOENER E P and H M FRANKEL, Effect of hyperthermia and P_aCO_2 on the slowly adapting pulmonary stretch receptor *Amer J Physiol* 1972 *222* 68—72

SCOTT F H On the relative parts played by nervous and chemical factors in the regulation of respiration *J Physiol* (Lond) 1908 *37* 301—326

Acta physiol scand 1974 92 351—363

From the Nobel Institute for Neurophysiology Karolinska Institutet and the Department of Automatic Control Royal Institute of Technology Stockholm Sweden

Steady State Effects of CO_2 and Temperature on the Relationship between Lung Volume and Inspiratory Duration (Hering-Breuer Threshold Curve)

By

G W Bradley[1] C von Euler Irja Marttila and B Roos

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Abstract

Bradley G W C von Euler I Marttila and B Roos *Steady state effects of CO_2 and temperature on the relationship between lung volume and inspiratory duration (Hering Breuer threshold curve)* Acta physiol scand 1974 92 351—363

The time course of the volume threshold curve for the inspiratory off switch i e the relationship between volume (V_T) and inspiratory duration (T_I) with intact vagus nerves was determined in steady conditions at different inspired CO_2 concentrations and body temperatures in lightly anesthetized cats by means of ramp shaped inflations and deflations of different rise times With intact vagal feedback the V_T—T_I data points could be very well fitted to hyperbolas in all conditions studied Changes in steady state F_ACO_2 never caused any detectable influence on the volume threshold curves Changes in body temperature in a range below the panting threshold caused pronounced effects on the volume threshold curve which could best be characterized as a shift of the whole curve along the volume axis to lower values with increased temperature Thus at each rate of volume expansion the threshold for inspiratory off switch was reached earlier the higher the temperature Also in the absence of vagal volume feed back T_I became correspondingly shorter with increased temperature which indicates that these temperature effects are mainly of central origin. The mechanisms for the CO_2 and temperature effects on tidal volume and inspiratory duration are discussed

In a preceding paper the relationship between tidal volume and inspiratory duration was studied during the transient part of the response to step changes in the CO_2 concentration of inspired gas both in the presence and absence of afferent vagal feed back of volume related information from the lungs (Bradley *et al* 1974 a) It was concluded that changes in PCO_2 may exert their effect on inspiratory duration not only by influencing the rate of increase of inspiratory activity and hence of the rate of expansion of the lungs (Clark and Euler 1972) but also by influencing another central control process giving an opposite effect on the timing of the in

[1] Present address Department for Respiratory Investigation Glasgow Royal Infirmary Glasgow Scotland

spiratory phase It appeared that these mutually opposing effects of CO_2 matched each other fairly well but could be somewhat differently affected by anesthetics and had different time courses in response to a step change in chemical drive These results called for a quantitative re investigation of the possible influence of CO_2 on the volume threshold curve under steady state CO_2 conditions Clark and Euler (1972) had shown only a qualitative similarity in the volume time relationships obtained during slow CO_2 accumulation and with artificial inflations

Changes in body temperature exert effects on ventilation which differ from those of the chemical drive factors by exerting a more specific influence on the rate determining mechanisms than on the tidal volume This was first described by Hey *et al* (1966) for man and has later been shown for the cat even in a range well *below the panting threshold* (Euler Herrero and Wexler 1970 Grundstein *et al* 1973) In order to gain some further information on the mechanisms determining rate and depth of breathing we have investigated the differences in effect between CO_2 and temperature on the Hering Breuer threshold curve and on the bulbopontine pace maker after vagotomy In our attempt to characterize these differences hyperbolic curves have been fitted to the experimental data and the effects of CO_2 and temperature on the parameters have been analysed As strongly emphasized by Clark and Euler (1972) the hyperbolic approximation may not necessarily be of any immediate physiological significance but serves merely as a convenient description of the curvilinear relationship between tidal volume V_T and inspiratory duration T_I However as will be discussed later in this and a subsequent paper (Bradley *et al* 1974 b) the parameters of the fitted equation may prove to have bearings on specific physiological functions within the neural mechanisms controlling rate and depth of breathing The neural correlate to the time course of the volume *threshold* curve has been elucidated in a recent paper on *the functional organization* within the solitary tract complex (Euler *et al* 1973)

Methods

Experimental procedures
The techniques used were essentially the same as those described in the preceding paper (Bradley *et al* 1974 a) The experiments were performed on 22 adult cats 18 of these being anesthetized with pentobarbitone and 3 with Dial and 1 decerebrated Great care was taken to keep the anesthetic level constant by using perfusion pumps for continuous infusion of anesthetics The body temperature could be raised using a heating pad and radient heat.

In order to determine the volume threshold curve by means of artificial inflations and deflations the respirator (Knox 1973) could be triggered from the integrated phrenic activity to provide either a positive or negative pressure at the tracheal cannula which increased or decreased in a ramp like form during the inspiration This either assisted or loaded inspiration The respirator was applied only every 4—6 breaths allowing P_ACO_2 to stay fairly constant. The breath immediately preceding this operation was used as control and other breaths were not used in the final computer analyses Negative pressure ramps were only applied to a degree which did not allow actual deflation of the lungs to occur Using this technique it was possible to delineate a V_T—T_I curve in 5—10 min under steady state condition of P_ACO_2 and temperature

In 4 experiments the cats were placed in a body plethysmograph (Euler *et al* 1970) to determine the possible effect of temperature and CO_2 on the functional residual capacity FRC This was achieved indirectly by measuring the expiratory reserve volume ERV (Euler and Fritts 1963)

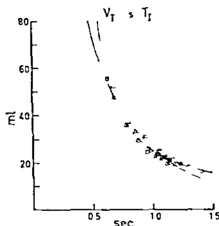


Fig 1 From a cat under light pentobarbitone anesthesia. Two different hyperbolic approximations fitted to the same V_T—T_I data according to eqns (1) and (3) respectively For the fit according to eqn (3) an optimal value for T_0 = 0 38 s was found Correlation coefficients Eqn (1) r = 0 928 eqn (3) r = 0 951

Data treatment

The off line computer treatment of data included cleaning procedures to exclude points arising from false triggering or augmented breaths (sighs) regression analysis between any combination of the different variables and special routines for the procedures to fit a hyperbola to V_T—T_I data

For most experiments the two parameter hyperbolic approximation of Clark and Euler (1972) has been employed

$$(V_T - V_0) \; T_I = C \qquad (1)$$

where V_0 and C are constants

This was achieved by transforming the T_I values to their reciprocals (T_I^{-1}) and applying ordinary linear regression analysis to fit the parameters C and V_0 to the corresponding straight line (with minimum mean square error MSQE)

$$V_T = C \; (T_I^{-1}) + V_0 \qquad (2)$$

The C and V_0 values describe the time course of the volume threshold curve The effects of changes in body temperature and end tidal CO_2 concentration may then be studied as changes in the parameters C and V_0 Changes in either parameter are more readily distinguished in a plot of V_T—$(T_I)^{-1}$ where C denotes slope and V_0 the intercept on the V_T axis (*cf* Fig 6 B and D)

In a few experiments the plotted V_T—T_I values suggested that the volume threshold curve might be better approximated by assuming a vertical asymptote at a positive T_I value (see Fig 1) This raised the question whether it was justified or not to use only two parameters in the descriptive expression of the V_T—T_I relationship or whether a third parameter T_0 ought to be introduced allowing a vertical asymptote at a T_I value other than zero in the hyperbola

$$(V_T - V_0)(T_I - T_0) = C \qquad (3)$$

Therefore a preliminary statistical analysis was performed by fitting three parameters to the V_T—T_I *data. Optimal T_0 values were found by a search procedure to minimize the MSQE* It was then found that

a) optimal T_0 (individual for each experiment) were normally positive but also negative values existed The average of all optimal T_0 values was about 200 ms
b) the mean square error in the data fit varied only little with T_0 around the optimal T_0 value
c) giving T_0 a value of zero did not reduce significantly the correlation coefficient,
d) no clear dependence or trend with changes in body temperature or PCO_2 could be seen when T_0 C and V_0 were plotted versus temperature or PCO_2 This was also verified by applying multiple regression analysis with temperature as independent variable
e) a strong coupling between T_0 C and V_0 was found
f) all attempts to reveal any trends for the three parameters with increasing body temperatures were unsuccessful in spite of the very obvious temperature effects seen in the primary V_T—T_I plots

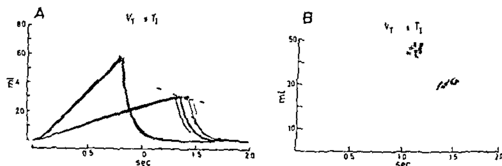


Fig 2 A and B from two different cats under light pentobarbitone anesthesia to show the scatter in inspiratory durations T_I (abscissa) and tidal volumes V_T (ordinate) at two different steady state alveolar CO_2 concentrations (F_ACO_2) A Superimposed inspiratory volume traces from 11 breaths at the lower F_ACO_2 (4 1 %) and 9 at the higher F_ACO_2 (6 7 %) The volume threshold curves dashed line was reached earlier at higher tidal volumes by the more rapidly expanding lung volume when the animal was breathing a CO_2 enriched gas than when breathing air The integrator providing the volume signal was reset to zero at the end of inspiration by the abrupt decrease in phrenic activity (see Methods) B Similarly obtained V_T—T_I data from another experiment The volume time courses were not recorded only the break points at the inspiratory off switch Triangles 43 breaths F_ACO_2 = 5 4 % T_I = 1 48 s±0 08 (S D) V_T = 31 7 ml±1 3 (S D) Squares 37 breaths F_ACO_2 = 6 . % T_I = 1 13 s±0 04 (S D) V_T = 46 4 ml±1 7 (S D)

It thus appeared unhelpful to introduce the T parameter in the fit since this increased the uncertainty in the other parameter values and obscured their possible temperature or CO_2 dependence

Results

Experimental considerations

The volume time relationship of individual breaths under steady state chemical conditions exhibited a certain amount of scatter However in the steady state the rate of volume expansion of the individual inspiratory phases showed relatively little variations and the scatter of the tidal volume V_T and inspiratory duration T_I values was *found to depend mainly on the fluctuations in* T_I the corresponding variations in V_T mainly appeared to be a consequence of the variations in T_I It was generally found that the scatter was bigger at low PCO_2 values *i e* when the rate of inspiratory volume expansion was slow than with high chemical drive This is shown in Fig 2 The corresponding expiratory durations T_E generally showed a linear dependence on the preceding T_I The scatter of the T_E values however was considerably bigger than that of T_I

The inflations used to obtain data in the uppermost part *of the volume range* were at times big and rapid enough to elicit so called augmented breaths or sighs (Reynolds 1962 Bendixen Smith and Mead 1964 Barlett 1971, Glogowska *et al* 1972) Such breaths showed higher V_T and larger T_I values than expected from the hyperbolic *range 2* characteristic of V_T—T_I relationship and gave *data in range 3* of Clark and Euler (1972) Values obviously falling in *range 3* were ex

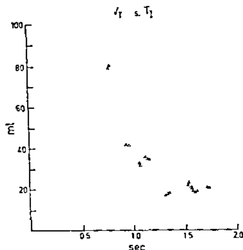


Fig 3 From a cat under light pentobarbitone anesthesia V_T—T_I data obtained with the inflation technique Note the apparently graded onset of range 3 (at volumes above 80 ml) F_ACO_2 4.9% body temperature 37.7 C

cluded from the analysis in the present work (see Methods) Because of the graded nature of augmented breaths it was difficult at times to delimit the border between *range 2* and *range 3* (see Fig 3) This may have influenced some of the V_T—T_I curves giving slightly too large T_I values in the upper volume range

The effect of anesthesia on ventilation is to depress tidal volume and respiratory rate The relationship between depth and rate of breathing however is not necessarily changed by the anesthetic as demonstrated by Hey *et al* (1966) In the present work it was confirmed that even marked changes in the level of pentobarbitone anesthesia often left the V_T—T_I relationship unaltered Nevertheless deep levels of anesthesia usually caused a disproportionate prolongation of T_I over V_T and over T_E (*cf* Bradley *et al* 1974 a) Great care was taken to maintain the level of anesthesia as constant as possible (see Methods) Sometimes however the volume threshold curve appeared to be moved to the left or downwards as the animal showed signs of arousal These shifts often occurred over such short periods of time that the tissue concentration of the anesthetic would probably not have changed significantly When such spontaneous shifts occurred during the course of an experiment the quantitative evaluation of the results was disturbed and when obvious discarded

The effect of CO_2 on the V_T—T_I relationship

With intact volume feed back a volume threshold curve (V_T—T_I curve) was obtained using the artificial inflation—deflation technique (see Methods) with the animal breathing air The animal was then given a CO_2 enriched gas mixture to breath When steady states had been reached the resulting V_T—T_I points were found to fall on the previously determined V_T—T_I curve for air This was studied on 13 cats and in no case could we detect any obvious deviation of the cluster of

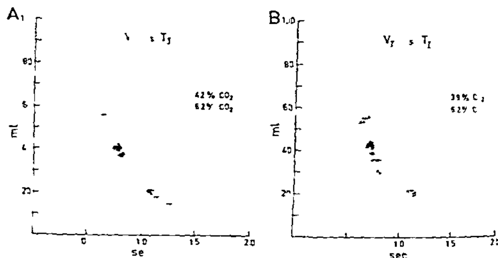


Fig 4 From a cat under light pentobarbitone anesthesia to show the lack of noticable effect of CO_2 on the volume threshold curves A The steady state V_T—T_I points (triangles) when breathing a CO_2 enriched gas (F_ACO_2 = 6 2 %) fall on the volume threshold points (crosses) obtained by the inflation technique when the cat was breathing air (F_ACO_2 = 3 9 %) B Two series of volume threshold points obtained by the inflation technique at two different steady state levels of F_ACO_2 (3 9 % crosses and 6 2 % triangles) show no detectable deviation from each other

points obtained with elevated CO_2 from the series of V_T—T_I points obtained on air An example of this is shown in Fig 4 A.

Volume threshold curves (V_T—T_I curves) using artificial inflations and deflations were also recorded from cats breathing different CO_2 mixtures and compared with those obtained when the cat was breathing air The series of inflation and deflation points on CO_2-curves were found to coincide very well with air-curves (Fig 4 B and 5) Comparisons of the parameters V_o C and T_o for the different data of eqn (3) showed that CO_2 had no systematic effect on any of these parameters (paired t test $p > 0.05$) If T_o was made zero and eqn (1) used there was still no effect on V or C to be found. The uncertainty in the hyperbolic regression curve of the V_T—T_I data and of the parameter values may be expressed in terms of volume In the ±1 S.D range this uncertainty corresponded to ± 1—2 ml. The correlation coefficients (r values) ranged between 0 92—0 94

This absence of any noticable effect of CO_2 on the V_T—T_I curves was true at all temperatures tested i e between 35 —41° C (Fig 5 A—B)

After vagotomy imposed inflations and deflations had no effect on T_I showing that under these conditions chest wall proprioceptors had no significant influence on the determination of T_I Repeating these procedures at various steady state levels of end tidal CO_2 concentrations usually showed a slight but varying shift towards smaller T_I values with increased CO_2 as described in a preceding paper (Bradley *et al* 1974 a) There was no sign of volume dependence on T_I at any CO_2 level

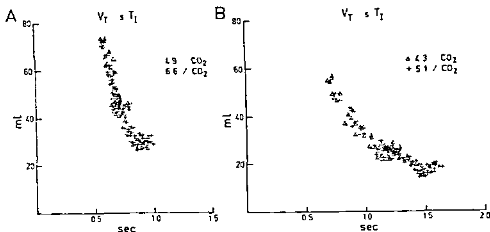


Fig 5 Similar to Fig 4 B From a cat under light pentobarbitone anesthesia to show the lack of noticable effect of CO_2 on the volume threshold curves at different body temperatures A Body temperature 39 6 —39 7 C Two series of volume threshold points at steady state F_ACO_2 = 4 9 % (triangles) and 6 6 % (crosses) B Body temperature 37 2 —37 5 C Two series of volume threshold points at steady state F_ACO_2 = 4 3 % (triangles) and 5 1 % (crosses)

The effect of temperature changes on the V_T—T_I relationship

With intact volume feed back from the lungs changes in temperature exerted a very marked effect on the Hering Breuer volume threshold curve (*i e* the V_T—T_I curve) displacing it closer to the axes and making it steeper at higher temperatures (*see* Fig 6) Similar effects have been described by Grunstein *et al* (1973) From a functional point of view this means that with the same rate of volume expiration *i e* with the same chemical drive inspiration was terminated at a lower volume threshold and at a shorter inspiratory duration the higher the body temperature The spontaneous scatter in the T_I values and the corresponding related variations in tidal volume were generally found to be smaller at higher than at lower temperatures

It proved possible to fit the V_T—T_I data obtained at different temperatures [illegible] hyperbolas according to eqn (1) with equally good fit for all temperatures [illegible] (r values 0 92—0 94)

If we employ a hyperbolic approximation effects on the V_T—T_I [illegible] be expressed by means of one of two main alternatives 1) either [illegible] the fitted V_T—T_I hyperbola along the volume axis *i e* a shift in the parameter V_0 with an unchanged C value or 2) as a change in the shape of the curve [illegible] parameter C with no change along the volume axis It was considered [illegible] possible functional significance to establish whether the temperature effects could be characterized as a change mainly in one or the other of these [illegible] parameters Fig 7 A—B show the parameters C and V extracted from a total of 47 different experiments plotted against their associated temperatures It [illegible] from [illegible]

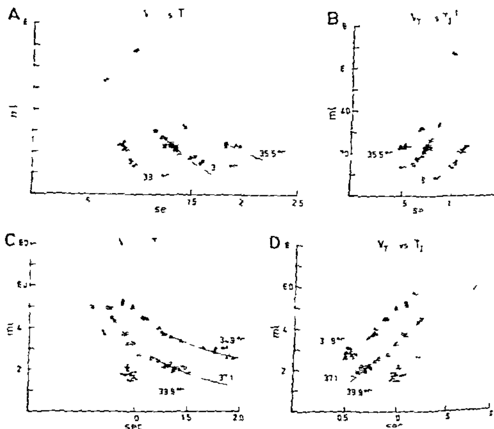


Fig. 6. From two different cats both under light pentobarbitone anesthesia. A. Volume threshold curves obtained by inflation technique at 3 different body temperatures. The regression lines correspond to eqn. (1) fitted with minimum mean square error. B. Tidal volume V_T plotted versus the reciprocal of inspiratory duration $(T_I)^{-1}$ for the same data as in A. The regression lines correspond to those in A. The slopes are defined by the parameter C of eqn (1) and the intercept on the V_T axis corresponds to V_o. The temperature effects correspond to a vertical shift of about 19 ml per °C. C and D similar to A and B but from another experiment. In this experiment the temperature responses correspond to a vertical shift of about 6.5 ml per °C.

figures that the individual C values vary considerably and that there is no significant trend in the change of the C-values with increased body temperature. Multiple regression analysis with temperature as the only independent input variable gave the correlation coefficients shown in Table I (obtained by IBM 360/75 computer BMDO2R program from UCLA).

The average V_o value obtained for each cat varies considerably between the different cats. Each cat, however, consistently showed decreasing V_o values with increasing body temperature as can be seen in Fig. 7 A. This trend was studied by computing for each cat the regression line and tabulating its slope. The slopes were then analysed with respect to mean, variance and standard error. The parameter C was also studied in this way and the results are summarized in Table II.

This elementary statistical treatment of the temperature effects suggests that the

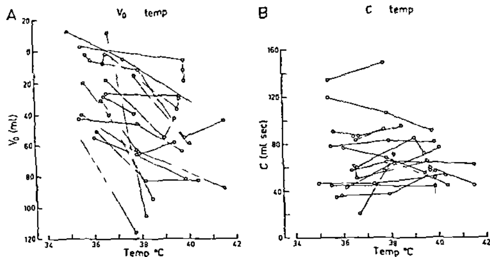


Fig 7 A The change of the V values with body temperature V_0 and C were obtained by fitting eqn (1) with minimum mean square error to V_T—T_I curves (from 9 cats) obtained by inflation technique at different body temperatures The data includes 16 cases (out of 47) where F_ICO_2 was increased to about 6 % The V values show a pronounced trend to decrease with increased body temperature B The change of the C values with body temp rature for the same data as in A There is no trend in changes of the C value

TABLE I Corr lation coefficients from multiple regress on analysis on C and V data with temperatu e as independent variables

C—temp	V —temp	C—V	Experimental cond tion
—0 081	—0 498	—0 469	31 cats breathing a r
—0 236	—0 300	—0 592	16 cats b eathing air with CO
—0 084	—0 496	—0 433	all 47 cats regardless of gas mixture breathed

TABLE II Changes in p rameters with temper ture mea ured as the slopes of the regression line for each experimental run Uncertainty given as standard error

Slope of C – temp ml s C	Slope of V – temp ml C	Experimental cond tion
0 7 0 8	—5 7±1 3	31 cats b eath ng air
2 8 3 0	—8 9±4 6	16 cats b eath ng a r with CO
1 6 – 1 5	—7 1±2 0	ll 47 cats reg rdless of gas mixture breathed

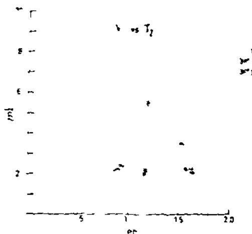


Fig 8 From a cat under light pentobarbitone anesthesia after bilateral vagotomy at 3 different body temperatures. Increased body temperature caused a marked shortening of T_I. Artificial inflations did not influence T_I

parameter C varies but little with temperature whereas the V_o parameter shows a significant trend to decrease with increased body temperature This means that the effect of a change in body temperature may best be described as a vertical shift of the whole V_T—T_I threshold curve whereas the curvature or shape is but little changed The described effects of temperature on the V_T—T_I curve was not changed in any significant way by giving the animal various CO_2 enriched gas mixtures to breath

With broken volume feed back after vagotomy Fig 8 shows that a rise in temperature after vagotomy caused a marked decrease in T_I confirming Grunstein *et al* (1973) However at all temperatures T_I was uninfluenced by artificially imposed volume changes

As stated above and in a previous paper (Bradley *et al* 1974 a) there was often some decrease of T_I with increased CO_2 especially with pentobarbitone This mismatch when present was not clearly influenced in any direction by changes in body temperature

Effects of temperature and CO_2 on FRC

In this investigation V_T has been used as a convenient index of changes in lung volume above FRC It has been assumed that there was no change in FRC with the experimental procedure This latter point has been tested by measuring ERV (as described in Methods) at different temperatures in 4 animals The results are tabulated in 2 temperature groups high (38 6° C—40 2° C) and low (35 0 C—37 0 C) A paired t test showed that temperature did not significantly influence ERV ($t = 0.9$ $\phi = 3$ and $p > 0.05$) and random changes in ERV were small (largest change was 6 0 ml) when compared with the change in V_o needed to shift the different temperature curves into apposition (typical shifts 10—20 ml)

Similarly it could be argued that although variations in P_ACO_2 had no apparent effect on the V_T—T_I curve it might possible exert changes in FRC which in turn could obscure a real change in central responsiveness to the inhibitory input from

the lungs This was investigated carefully in one animal by measuring ERV immediately before during and after breathing CO_2 enriched gas mixture Increasing the P_ACO_2 had no consistent effect on the ERV (paired t test $t = 1\ 9$ $\phi = 3$ and $p > 0\ 05$)

Discussion

The aim of this paper has been to define the influence of CO_2 and temperature changes on the tidal volume—inspiratory duration relationship In discussing the results we will first consider the limitations in terms of scatter and variability in the data

With constant chemical drive for ventilation the rate of increase of inspiratory activity and thus of lung volume expansion in each inspiration was fairly constant and subject to less spontaneous variations than the durations of inspiration and expiration This scatter in the V_T—T_I relationship with steady state conditions of chemical drive is thus mainly distributed in a direction crosswise to the volume threshold curve (*cf* Newsom Davis 1974) This implies that noise variations in the duration of inspiratory activity will cause corresponding variations in tidal volume (and in expiratory duration) so that the instantaneous ventilation stays relatively constant (Priban 1963 Euler *et al* 1970 Euler 1974) The observation that this type of scatter was usually bigger at the low end of the volume threshold curve than at the high volume range suggests that with slow increase in inspiratory inhibitory neural activity the threshold of the switch mechanism is approached slowly and a little random noise superimposed on the off switch mechanism ensures a relatively large variability in the termination of inspiration At high PCO_2 on the other hand the threshold is approached more rapidly and steeply and noise will therefore have less influence on the timing of inspiratory duration

Another type of scatter which limits the accuracy with which changes in the V_T—T_I relationships can be defined appears to be caused by fluctuations in the general state of arousal which may be accompanied by shifts of the volume threshold curve The precise relationship between the excitatory state of brain stem reticular structures and the factors determining the respiratory pattern is virtually unknown

CO_2 effects Our results would indicate that CO_2 does not exert an influence on the volume threshold curve which could be detected in the presence of the scatter in our data This was true at all body temperatures in the range tested In the preceding paper (Bradley *et al* 1974 a) it was argued however that after vagotomy when there was no volume effect at all on the inspiratory duration there was often a slight but variable decrease of inspiratory duration with CO_2 also as a steady state response It has been argued that this may be due to changes in chemical drive exerting two opposing effects on the timing of inspiratory duration which do not cancel out perfectly especially not after pentobarbitone (Bradley *et al* 1974 a) This raises the question why such a mismatch when evident after vagotomy is not clearly seen in the V_T—T_I curve with intact volume feed back It seems very likely

however that the better match achieved with intact vagal nerves than after vagotomy is due to the slight depressing effect of increased PCO on the pulmonary stretch receptor function (Mustafa and Purves 1972 Schoener and Frankel 1972) These points will be further discussed in a subsequent paper (Bradley *et al* 1974 b)

Temperature effects The general effect of a moderate increase in body temperature was to lower the volume threshold for the inspiratory off switch so that at any particular time smaller tidal volumes or artificial volume increments were required to reach the threshold for inspiratory arrest We did not find any sign of a volume independent range (*range I*) in thermal tachypnoea as was found for the rapid breathing induced by urethane (Clark and Euler 1972) Also after vagotomy the inspiratory phase got progressively shorter with increasing body temperature These results are in general agreement with those of Grunstein *et al* (1973)

In some contrast to the report by Grunstein *et al* (1973) we found in the case of intact vagus nerves that the effect of a temperature rise can best be described merely as a shift of the volume threshold curve downwards along the volume axis without any marked change in the shape of the curve These shifts in V_T—T_I relationships were equivalent to volume changes in the order of −5 to −18 ml per 1° C This is in slight variance with Grunstein *et al* (1973) who reported on a change in shape as well The difference may depend on the fact that these authors have only investigated tidal volumes below the normal physiological range ie between zero magnitude (complete tracheal occlusion at FRC) and the eupnoeic value The V_T—T_I data in our studies has covered a range of ventilation from eupnoeic to maximal By applying negative pressures during the experimental trial we have extended our volume range below eupnoeic tidal volume However in doing so we often obtained results suggesting that the big loads necessary to reduce tidal volumes to values comparable to those studied by Grunstein *et al* (1973) elicit more complex reflex effects than merely the reduction of the vagal inspiratory inhibition

Grunstein *et al* (1973) have demonstrated a close relationship between the temperature effects on T_I obtained in the presence and in the absence of volume feed back from the lungs This indicates that the temperature effects under discussion are largely of central origin (Euler *et al* 1970) and cannot be explained on basis of the thermal effect on the discharge rate of pulmonary stretch receptors reported by Schoener and Frankel (1972) However these thermal effects on the receptor properties (so far only studied in rats) should be taken into account in any quantitative consideration of the control system (Bradley *et al* 1974 b)

In a recent paper from this laboratory (Euler *et al* 1973) it was postulated that the combined vagal and central inspiratory excitatory (CIE) activity of a pool of solitary tract neurons project onto the inspiratory off switch mechanism which executes the termination of inspiration when a certain threshold is reached The effects of temperature changes both in the presence and absence of vagal feed back could be well explained by assuming e g that the threshold for inspiratory off switch is influenced by an input from the hypothalamic thermoceptive structures in

such a way that an increasing hypothalamic temperature would reduce the threshold. Consequently the threshold would be reached earlier at higher temperature both in the case with intact vagus nerves (by the combined vagal and CIE activity) and in the absence of volume feed back (by the CIE activity alone). It was postulated above and in the preceding paper (Bradley *et al.* 1974 a) that this off switch threshold is one of the two main factors through which changes in PCO_2 influence the depth and rate of breathing in addition to its matching effect on the rate of increase of volume expansion.

The off switch mechanism and the effects exerted by CO_2 and changes in body temperature will be discussed in further detail in a subsequent paper (Bradley *et al.* 1974 b).

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References

BARTLETT, D. JR. Origin and regulation of spontaneous deep breaths. *Resp. Physiol.* 1971. *12*. 230—238.

BENDIXEN, H. H., G. M. SMITH and J. MEAD. Pattern of ventilation in young adults. *J. appl. Physiol.* 1964. *19*. 195—198.

BRADLEY, G. W., C. VON EULER, I. MARTTILA and B. ROOS. Transient and steady state effects of CO_2 on mechanisms determining rate and depth of breathing. *Acta physiol. scand.* 1974 a. *92*. 341—350.

BRADLEY, G. W., C. VON EULER, I. MARTTILA and B. ROOS. A model of the reflex inhibition of inspiration in the cat. *Kybernetik.* 1975 b. In press.

CLARK, F. J. and C. VON EULER. On the regulation of depth and rate of breathing. *J. Physiol.* (Lond.) 1972. *222*. 267—295.

EULER, C. VON. Time dependent changes in responsiveness to inflations and deflations of the lungs during inspiration and expiration: their significance for the control of depth and rate of breathing. *Arch. Fisiol.* 1972. *69*. Suppl. 424—432.

EULER, C. VON and H. W. FRITTS JR. Quantitative aspects of respiratory reflexes from the lungs and chest walls of cats. *Acta physiol. scand.* 1963. *57*. 284—300.

EULER, C. VON, F. HERRERO and I. WEXLER. Control mechanisms determining rate and depth of respiratory movements. *Resp. Physiol.* 1970. *10*. 93—108.

EULER, C. VON, J. N. HAYWARD, I. MARTTILA and R. J. WYMAN. Respiratory neurones of ventrolateral nucleus of the solitary tract of cat: vagal input, spinal connections and morphological identification. *Brain Res.* 1973. *61*. 1—2?.

GLOGOWSKA, M., P. S. RICHARDSON, J. W. WIDDICOMBE and A. J. WINNING. The role of the vagus nerves, peripheral chemoreceptors and other afferent pathways in the genesis of augmented breathing in cats and rabbits. *Resp. Physiol.* 1972. *16*. 179—196.

GRUNSTEIN, M. M., W. M. FISK, L. A. LEITER and J. MILIC EMILI. Effect of body temperature on respiratory frequency in anesthetized cats. *J. appl. Physiol.* 1973. *34*. 154—159.

HEY, E. N., B. B. LLOYD, D. J. C. CUNNINGHAM, M. G. M. JUKES and D. P. G. BOLTON. Effects of various respiratory stimuli on the depth and frequency of breathing in man. *Resp. Physiol.* 1966. *1*. 193—205.

KNOX, C. K. Characteristics of inflation and deflation reflexes during expiration in the cat. *J. Neurophysiol.* 1973. *36*. 284—295.

MUSTAFA, M. E. K. Y. and M. J. PURVES. The effect of CO_2 upon discharge from slowly adapting stretch receptors in the lungs of rabbits. *Resp. Physiol.* 1972. *16*. 197—212.

NEWSOM DAVIS, J. and D. T. STAGG. Analysis of breathing patterns in man. *Arch. Fisiol.* 1972. *69*. Suppl. 418—423.

PRIBAN, I. P. An analysis of some short term patterns of breathing in man at rest. *J. Physiol.* Lond. 1963. *166*. 425—434.

REYNOLDS, L. B. JR. Characteristics of an inspiration augmenting reflex in anesthetized cats. *J. appl. Physiol.* 1962. *17*. 683—688.

SCHOENER, E. P. and H. M. FRANKEL. Effect of hyperthermia and PCO_2 on the slowly adapting pulmonary stretch receptor. *Amer. J. Physiol.* 1972. *222*. 68—72.

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From the Institute of Physiology University of Bergen Norway

Single-Nephron Filtration during Hemorrhagic Hypotension in the Conscious Rat

By

GUNNAR CLAUSEN and INGVALD TYSSEBOTN

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Abstract

CLAUSEN G and I TYSSEBOTN *Single nephron filtration during hemorrhagic hypotension in the conscious rat* Acta physiol scand 1974 92 364—373

To decide whether redistribution of filtrate between superficial (S) and deep (D) nephrons takes place when GFR is reduced by hemorrhagic hypotension (HH) the amount of filtered ^{14}C ferrocyanide precipitated as Prussian blue in single S and D nephrons was determined in rats during HH of 80 65 and 50 mm Hg mean arterial pressure ($\overline{AP}$) Marked filtration and urine flow persisted only in the $\overline{AP}$ 80 mm Hg group whereas the major fraction of nephrons had ceased filtration in four out of five rats at $\overline{AP}$ 50 mm Hg The distribution of radioactivity between S and D proximal tubules was not altered in any of the HH groups as compared to normotensive controls

During hemorrhagic hypotension (HH) renal blood flow and filtration pressure may fall to levels where urine flow becomes zero This does not necessarily imply complete cessation of glomerular filtration According to Brenner Troy and Daugharty (1971) a net filtration pressure of approximately 10 mm Hg is sufficient to account for the normal rate of filtration Formation of filtrate might therefore take place during HH even at very low mean arterial pressure provided preglomerular resistance is reduced through autoregulatory mechanisms Thus anuria during HH might be caused by an almost complete tubular reabsorption of ultrafiltrate On the other hand GFR might be zero due to lack of such dilatation or even to constriction of all or some afferent arterioles because of sympathetic nervous activation or increased levels of circulating vasoconstrictor agents

If filtration does take place it may be a) proportionately reduced in all nephrons b) disproportionately reduced among nephrons resulting in a larger functional heterogeneity or c) reduced to different extent in superficial or deep nephrons

The only previous information on filtrate distribution in HH rats is available from preliminary reports by Coelho *et al* (1970) and Carriere *et al* (1972) who

found a similar reduction of filtration rate in deep and superficial nephrons in rats bled to mean arterial pressures of 65 and 70 mm Hg

Several studies during HH in dogs indicate a selective vasoconstriction in the outer cortex (Carriere *et al* 1966 Truniger Rosen and Oken 1966 Logan *et al* 1971) suggesting greater reduction of GFR in superficial than in deep glomeruli Other investigators have observed a fairly proportional fall in blood flow in all areas of the renal cortex suggesting a proportional reduction of deep and superficial GFR (Deetjen and Kramer 1962 Aukland and Wolgast 1968 Aukland *et al* 1973 Ofstad Willassen and Egenberg 1973) However by all these methods blood flow from areas containing many glomeruli has been measured. Thus none of these previous studies would reflect possible increased heterogeneity of single glomerular blood flow or filtration

We therefore decided to investigate whether HH is associated with an altered distribution of single nephron GFR by means of the ferrocyanide technique (Hanssen 1958 1962) which allows measurement of relative filtration rate of single nephrons determined without blood and urine samples even in kidneys not producing urine

Methods

The experiments were made on Albino Wistar rats weighing 240—400 g The rats had free access to water and food before the experiment A PE 90 catheter with sideholes was inserted into the urinary bladder through a small suprapubic incision. A femoral artery and vein were cannulated by PE 50 catheters for recording of mean arterial pressure ($\overline{AP}$) and for infusion of inulin respectively These preparations were made in about 30 min during ether anesthesia. The rats were placed in a restraining cage where they recovered completely from anesthesia as previously described (Clausen and Tyssebotn 1973) 1 h after surgery 200 I U heparin was injected i

A primary dose of 50 μl/100 g BW and a sustaining infusion of 5 μl/min 100 g BW of 4 inulin in isotonic saline was given i v 3 consecutive 60 min clearance periods were completed in the control group before sacrifice whereas two 60 min periods preceded the bleeding in the HH groups Plasma inulin was measured in 0 5 ml midperiod blood samples and in urine by the anthrone method (Fuhr Kaczmarczyk and Kruttgen 1955) After the two 60 min clearance periods the inulin infusion was stopped and the HH rats were bled from the femoral artery to the desired mean arterial pressure level in the course of 10 to 15 min The pressure was held constant for 2 h by bleeding or reinfusion of small amounts of shed blood at 50 65 and 80 mm Hg in HH groups I (5 rats) II (4 rats) and III (2 rats) respectively In all groups 150 μl of 20 % sodium ferrocyanide solution containing 150 μCi ^{14}C labelled ferrocyanide was injected into the femoral vein in the course of 2 s The single injection ferrocyanide method requires that renal blood flow and filtration are abruptly arrested before the filtered ferrocyanide reaches the loop of Henle This was obtained by guilloting the rats just below the diaphragm through a slit in the cage The rats were thus killed 8—10 s after injection in controls and in HH group III showing significant urine flow whereas a delay of 20—5 s was used in the other 2 HH groups where anuria suggested greatly reduced GFR These two time intervals ensured optimal proximal tubular radioactivity and that filtered ferrocyanide remained within 2/3 of the proximal tubular length in all nephrons

The kidney was rapidly excised cut in transverse 2—3 mm thick slices and frozen immediatel Preparation of slices microdissection of tubules and determination of tubular radioactivit were made as previously described (Clausen and Tyssebotn 1973) The radioactivity was determined in single proximal tubules with attached glomerulus and broken at the beginning of the loop of Henle Distal tubular segments corresponding to proximal tubular length served as blanks, representing ferrocyanide of extratubular origin adhering to the tubular outside i e non filtered ferrocyanide Net proximal radioactivity proximal minus distal activity is proportional to the relative individual glomerular filtration rate (gfr The gfr was calculated in per cent of the average net radioactivity of deep nephrons

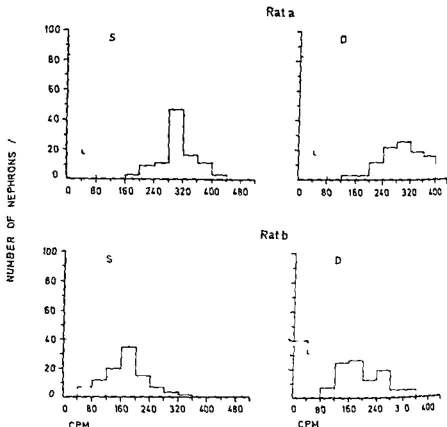


Fig 1 Normotension $\overline{AP}$ 126 mm Hg Distribution of radioactivity (cpm) in superficial (S) and deep (D) proximal tubules (solid line) and in distal tubular segments (dotted line) Ordinate Number of nephrons (72 of each type) in per cent of total

The nephrons were divided in two groups Superficial (S) nephrons include those having tightly folded proximal convolutions and a well defined straight proximal segment The deep (D) nephrons defined as lacking both these characteristics Intermediates were discarded By this definition the D nephron group includes both juxtamedullary nephrons and nephrons without vasa rectae but it is not possible to differentiate between these nephron types by the present method Usually 9 tubules 3 distal 3 S and 3 D proximal were dissected from each of 15 different sectors of slices from each rat giving 45 tubules of each type The sampling of tubules were made with a blue filter on the microscope that made the Evans blue invisible

Three rats maintained at $\overline{AP}$ 50 mm Hg showed sudden heavy struggling with an abrupt rise in arterial blood pressure to above 100 mm Hg Further bleeding in order to regain the desired $\overline{AP}$ gave a rapid drop in $\overline{AP}$ to very low levels Reinfusion of shed blood had no effect and the rats died within seconds These 3 rats were excluded from the material

Results

When recovered from anesthesia the restrained rats were calm accepted water but not food During bleeding most of the rats remained calm but some struggled for a brief period when the $\overline{AP}$ was passing 80—60 mm Hg

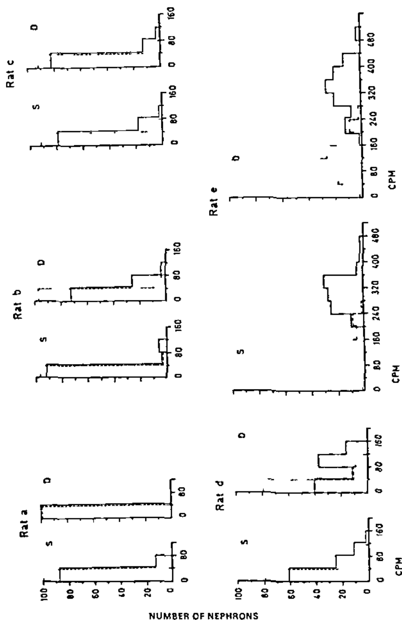


Fig. 2 Hemorrhagic hypotension $\overline{AP}$ 50 mm Hg. Distribution of radioactivity (cpm) in superficial (S) and deep (D) proximal tubules (solid line) and in distal tubular segments (dotted line). Ordinate: Number of nephrons (45 of each type) in per cent of total

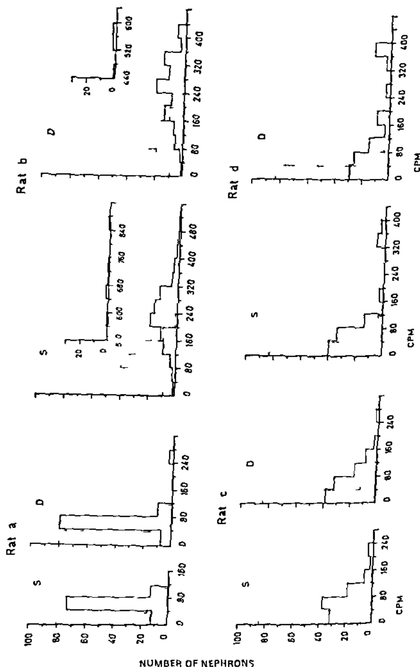
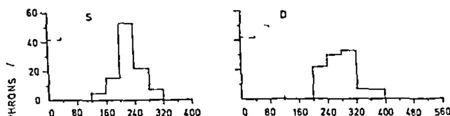


Fig 3 Hemorrhagic hypotension $\overline{AP}$ 65 mm Hg Distribution of radioactivity (cpm) in superficial (S) and deep (D) proximal tubules (solid line) and in distal tubular segments (dotted line) Ordinate Number of nephrons (45 of each type) in per cent of tot.

Rat a



Rat b

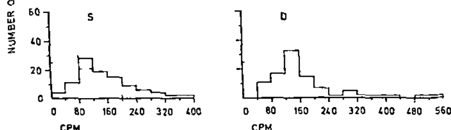


Fig 4 Hemorrhagic hypotension $\overline{AP}$ 80 mm Hg Distribution of radioactivity (cpm) in superficial (S) and deep (D) proximal tubules (solid line) and in distal tubular segments (dotted line) Ordinate Number of nephrons (45 of each type) in per cent of total

In control periods $\overline{AP}$ remained stable at level between 120 and 135 mm Hg The mean inulin clearance of the different groups varied between 0 82 and 1 15 ml/min 100 g BW and the urine flow between 5 and 25 μl/min 100 g BW The diuresis however ceased during HH in groups I and II

The control group consisting of 6 rats is identical to that used in a previous study (Clausen and Tyssebotn 1973) where 18 nephrons of each type were dissected from each rat The average proximal radioactivity in the control group was 3—8 times higher than the average distal tubular blank clearly indicating glomerular filtration In the control group the average S gfr was 90 (S E ±3) % of D gfr About 6 months after the first series was dissected from this group the number of dissected tubules was increased to 72 in 2 of the control rats in order to provide more information on gfr distribution. These 2 rats (Fig 1) present the higher and lower values of S gfr in the control group In rat a the average amount of filtered ferrocyanide was equal in D and S nephrons whereas rat b showed 19 % lower filtration rate in S than in D nephrons

As evident from Fig 1 b about 15 % of the S and 5 % of the D nephrons contained less radioactivity than the highest tubular blanks This overlaping does not however mean that all these nephrons were nonfiltering It is not possible to estimate the exact fraction of nonfiltering nephrons by the present procedure and the overlaping therefore indicates only the maximal fraction of such nephrons


Fig 5a


Fig 5b

Fig 5 *a* Patchy distribution of Prussian blue in hemorrhagic hypotension ($\overline{AP}$ 50 mm Hg) Same rat as in Fig 2 a. *b* Distribution of Prussian blue in a normotensive rat

At $\overline{AP}$ 50 and 65 mm Hg (HH group I and II) proximal radioactivity varied from control group values to those of distal tubules *i e* to zero detectable filtration The major fraction of both D and S nephrons seemed to be nonfiltering in four rats (Fig 2 a b c Fig 3 a) Filtration probably remained in some nephrons in 3 rats (Fig 2 d Fig 3 c d) whereas filtration seemed to be preserved in most nephrons in two rats (Fig 2 e Fig 3 b) The average proximal radioactivity was similar in D and S nephrons in all animals indicating that filtrate distribution between the two types of nephrons was not markedly different from that of normotensive animals In some of the HH rats the variation of proximal radioactivity was considerably greater than in controls (Fig 3 b c d) suggesting an increased variation of filtration rate in both nephron types

The results from the two rats at $\overline{AP}$ 80 mm Hg (HH group III) are shown in Fig 4 Avarage proximal activity was not markedly different from that observed in normotensive rats and most nephrons appeared to be filtering Average S gfr was about 10 % lower than D gfr indicating a distribution of filtration equal to that of the control group However the scatter of gfr in one of these rats (Fig 4 b) was considerably greater than in controls

The cortical Prussian blue staining was homogenous in the controls and most of the HH rats As demonstrated in Fig 5 however a macroscopic patchy distribution of Prussian blue was observed in slices from 3 rats at AP 50 mm Hg These rats (Fig 2 a, b, c) had no detectable filtration

Discussion

2 rats bled to 50 and 65 mm Hg had as much ferrocyanide in proximal tubules as control animals (Fig 2 e Fig 3 b) Since the time allowed for filtration of ferro cyanide in these rats was roughly three times that of the controls one might assume that filtration of ferrocyanide was one third of control level This assumption may however be completely wrong Equal amounts of ferrocyanide were injected in all rats and if the renal fraction of cardiac output is unchanged which may well be the case during HH (Netze Wyler and Rudolph 1968) the amount of ferrocyanide filtered should be proportional to the filtration fraction provided the time from injection to arrested blood flow was sufficient for the whole bolus to pass through the glomeruli Thus reduction of GFR to 10 % of control level with a concomitant reduction of renal plasma flow to 20 % should result in only 50 % reduction of the amount of filtered ferrocyanide It is therefore obvious that the proximal tubular net activity gives very limited information on absolute filtration rate However when proximal tubular activity is close to that of corresponding lengths of distal tubules it seems safe to conclude that there has been negligible filtration The proximal activity approached that of distal tubules with increasingly severe hypo tension and was practically identical in 3 animals at $\overline{AP}$ 50 mm Hg (Fig 2 a b c) This finding corroborates the use of distal tubules to correct for ferrocyanide brought to the proximal tubules by peritubular blood and not by filtration and agrees well with previous results obtained in rats where GFR was stopped by ureteral ligation (Coehlo *et al* 1971) or by aortic clamping (Morris *et al* 1972)

Since the extratubular radioactivity was not the same in all nephrons as indicated by the range of activity in the distal blank it is not possible to decide whether a borderline value of a given proximal tubule does represent filtration or not It would still seem feasible to make a statistical evaluation of the fraction of filtering nephrons but attempts with several methods did not seem to give adequate estimates For this reason we choose to present the raw data in Fig 1 2 3 and 4 giving a fairly good visual impression of the presence of glomerular filtration and its distri bution Since none of the kidneys with patchy distribution of ferrocyanide pre cipitated as Prussian blue produced detectable amounts of filtrate the patching must represent nonfiltered material possibly due to heterogeneously distributed blood flow and/or pooling of blood in peritubular capillaries This interpretation is compatible with the results of Truniger Rosen and Oken (1966) and Carriere *et al* (1966) who demonstrated patchy blood flow distribution in HH dogs by [8] Kr auto-radiography An alternative explanation might be uneven washout of extravascular ferrocyanide possibly due to a patchy distribution of extracellular fluid in the kidney during HH

The present results do not indicate any marked redistribution of filtrate during light or severe hemorrhagic hypotension These observations agree well with results obtained by the same technique by Coehlo *et al* (1970) and Carriere *et al* (1972) showing proportional fall in S and D nephron GFR in HH rats at $\overline{AP}$ 50 mm Hg

During stepwise lowering of the perfusion pressure by constriction of aorta some investigators using micropuncture technique have found a slightly greater fall in total GFR than in superficial nephron filtration rate (Landwehr *et al* 1968 Arrizurieta *et al* 1969, Rodicio *et al* 1969), whereas others have reported a greater fall in superficial GFR than in total GFR (Brenner *et al* 1968) Bonvalet Bencsath and de Rouffignac (1972) using Hanssen s ferrocyanide and micropuncture methods found unaltered single nephron GFR down to $\overline{AP}$ 90 mm Hg Further reduction of $\overline{AP}$ lowered the superficial and deep nephron GFR proportionally down to $\overline{AP}$ 40 mm Hg where the filtration rate was zero Morris *et al* (1972) concluded from micropuncture studies that only 1 % of the nephrons filtered at $\overline{AP}$ 40 mm Hg The present results indicate that filtration had ceased at $\overline{AP}$ 50 mm Hg in 4 out of 5 rats and in 1 out of 4 at $\overline{AP}$ 65 mm Hg *i e* 10—25 mm Hg higher than in the aortic constriction experiments This difference may be due to sympathoadrenergic activation and release of vasopressin during HH, causing renal vasocon triction, whereas aortic clamping leads to autoregulatory vasodilation Heterogeneously distributed vasoconstriction and reduced blood flow is compatible with the great variation in distal and proximal tubular radioactivity in filtering kidneys However the results do show that filtration is proportionately reduced in deep and superficial nephrons during HH of about 2 h duration and thus suggest a similar reduction of blood flow

References

ARRIZURIETA MUCHNIK E E W E LASSITER E A LIPHAM and C W GOTTSCHALK Micropuncture study of glumerulo tubular balance in the rat kidney *Nephron* 1969 *6* 418—436

ALKLAND K and M WOLGAST Effect of hemorrhage and retransfusion on intrarenal distribution of blood flow in dogs *J clin Invest* 1968 *47* 488—501

ALKLAND K A KIRKEBO E LOYNING and I TYSSEBOTN Effect of hem rrhagic hypotension on the distribution of renal cortical blood flow in the anaesthetized dog *Acta physiol scand* 1973 *87* 514—525

BRENNER B M C M BENNETT and R W BERLINER The relationship between glomerular filtration rate and sodium reabsorption by proximal tubule of the rat nephron *J clin In est* 1968 *47* 1358—1374

BRENNER B M J L TROY and T M DAUGHARTY The dynamics of glomerular ultrafiltration in the rat *J clin Invest* 1971 *50* 1776—1780

BONVALET J P P BENCSATH and G DE ROUFFIGNAC Glomerular filtration rate of superficial and deep nephrons during aortic constriction *Amer J Physiol* 1972 *22?* 55—606

CARRIERE S G D THORBURN C C C O'MORCHOE and A C BARGER Intrarenal distribution of blood fl w in dogs during hemorrhagic hypotension *Circulat Res* 1966 *19* 167—179

CARRIERE S J P BONVALET P BENCSATH P BOULET and C DE ROUFFIGNAC Single nephron glomerular filtration rate (SNGFR) during hemorrhagic hypotension in rat *Circ Res* 1972 *806* Abstract

CLAUSEN G and I TYSSEBOTN Intrarenal distribution of glomerular filtration in conscious rats during saline infusion *Acta physiol scand* 1973 *89* 289—295

COEHLO J B K C H CHIEN S R STELLA and S E BRADLEY Relationship of extraluminal tubular deposition of ferrocyanide to peritubular perfusion rate in cortical and medullary nephron segments of the rat kidney *Circulat Res* 1971 *29* 21—28

COEHLO J B S R STELLA K C HUCHIEN and S E BRADLEY Glomerular filtration rate (GFR) in superficial (S and juxtamedullary (JM) nephrons during salt loading and hemorrhagic hypotension *Clin Res* 1970 *18* Abstract

FUHR J J KACZMARCZYK and C D KRÜTTGEN Eine einfache colorimetrische Metode zur Inulinbestimmung fur Nieren Clearance Untersuchungen bei Stoffwechselgesunden und Diabetikern *Klin Wschr* 1955 *33* 729—730

HANSSEN O E A histochemical method for evaluation of excreted sodium ferrocyanide in isolated tubules of the mouse kidney *Acta path microbiol scand* 1958 *44* 363—371

HANSSEN O E Method for comparison of glomerular filtration in individual rat nephrons *Proc 2nd Int Nephrol Congr* 1963 pp 527—529

KRAMER, K. Renal failure in shock. In Bock K. D (ed) *Shock pathogenesis and therapy* Springer Verlag Berlin 1962 134—144

LANDWEHR D M J SCHNERMANN R M KLOSE and G GIEBISCH Effect of reduction in filtration rate on renal tubular sodium and water reabsorption *Amer J Physiol* 1968 *215* 687—695

LOGAN A P JOSE G EISNER L LILIENFIELD and L SLOTKOFF Intracortical distribution of renal blood flow in hemorhagic shock in dogs *Circulat Res* 1971 *29* 257—265

MORRIS G R E A ALEXANDER, F J BRUNS and N G LEVINSKY Restoration and maintainance of glomerular filtration by mannitol during hypoperfusion of the kidney *J clin Invest* 1972 *51* 1555—1564

OFSTAD J Y WILLASSEN and K E EGENBERG Distribution of radioisotope labelled microparticles in the renal cortex of dogs in hemorrhagic hypotension *Scand J clin lab Invest* 1973 *31* 277—287

RODICIO J J HERRERA ACOSTA J C SELLMAN F C RECTOR JR. and D W SELDIN Studies on glomerulotubular balance during aortic constriction ureteral obstruction and venous occlusion in hydropenic and saline loaded rats *Nephron* 1969 *6* 437—456

TRUNIGER B S M ROSEN and D E OKEN Renale Hamodynamikk und hamorrhagische Hypotension *Klin Wschr* 1966 *44* 857—862

Acta physiol scand 1974 92 374—390

From the Department of Clinical Neurophysiology University Hospital Uppsala Sweden

Afferent C Units Responding to Mechanical, Thermal and Chemical Stimuli in Human Non-Glabrous Skin

By

H Erik Torebjörk

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Abstract

Torebjörk H E *Afferent C units responding to mechanical thermal and chemical stimuli in human non glabrous skin* Acta physiol scand 1974 92 374—390

C unit activity was recorded with microelectrodes from intact sensory fascicles in the human peroneal nerve The analysis includes 46 afferent units with receptive fields predominantly on the dorsum of the foot and ankle 16 units were tested quantitatively Another 30 units were tested qualitatively by a combination of electrical and natural stimuli in the skin This method was valuable for a reliable identification of activity in individual afferent C elements when several C units with similar potential amplitudes responded to testing in the skin The units were not spontaneously active at normal skin temperatures but one unit showed a low frequency discharge at a skin temperature of 22 C Cooling by ether evaporation was an inefficient stimulus except for 2 units Gentle mechanical stimuli did not activate any of the units whereas afferent C unit impulses were induced by moderately intense mechanical stimuli noxious heat and various chemical irritants The sensations produced by stimuli inducing intense afferent C unit activity were reported as burning or delayed pain whereas stimuli eliciting low frequency activity often were reported as itch It is concluded that polymodal C receptors similar with polymodal nociceptors in the cat and monkey are numerous in skin areas sparsely covered with hairs on the dorsum of the foot and ankle in man whereas no low threshold C mechanoreceptors were identified The experiments do not exclude the possibility that both delayed pain and burning itch sensations may be mediated by different impulse patterns from polymodal C receptors

It was recently shown that single C unit activity can be recorded with microelectrodes percutaneously inserted into intact cutaneous nerves in alert man (Torebjörk and Hallin 1970) Using this technique the receptive properties of a number of afferent C units have been tested in the dorsum of the human hand (Hallin and Torebjörk 1970a van Hees and Gybels 1972 Torebjörk and Hallin 1974a) These C units responded most intensively to firm mechanical stimuli and noxious heat and activity was also elicited by chemical stimuli such as histamine In several respects the responses of afferent C units in man were similar to responses in polymodal nociceptors in the cat (Bessou and Perl 1969) Low threshold C mechanoreceptors activated by touch stimuli and mild cooling which are common in hairy skin in the cat (Bessou *et al* 1971) have as yet not been identified in man However only small

numbers of C units have been investigated in man due to difficulties in identification of afferent C units in human nerves

In this investigation the interest was focused on the following questions

1 Is it possible with improved techniques to increase the probability for identification of afferent C units in intact human cutaneous nerves?

2 What are the receptive properties of those afferent C receptors which are identified by their responses to mechanical stimuli in the dorsum of the foot and ankle?

3 What sensations are produced by stimuli capable of exciting afferent C units?

Methods

Subjects The investigations were carried out on 8 males ranging in age from 25 to 35 years They had no signs or symptoms of any neurological o dermatological disease

General procedure The subjects sat relaxed in a chair with the examined extremity comfortably supported Lacquer insulated tungsten microelectrodes were inserted manually into the nerve to be explored

Technical details The electrodes (0 2 mm in diameter with a tapered portion about 2 mm long and a sharp exposed tip seldom exceeding 30 μm) and the recording and display system were described in previous reports (Vallbo and Hagbarth 1968 Hagbarth *et al* 1970) The signal to-noise ratio of the C potentials was improved by bandwidth reduction from 0 2—10 kHz to 0 2—2 kHz (Torebjork and Hallin 1974 a) and the signals were sometimes fed through an amplitude discriminator adjusted to eliminate 50—75 % of the remaining noise (Hagbarth *et al* 1970) Signals distorted by filtering are not calibrated in the figures In studying the C responses evoked by combined electrical and natural stimuli the C unit responses were represented as dots and successive responses displayed below each other to present changes in latency in a compact form (Torebjork and Hallin 1974 b) An instantaneous frequency plot was used to measure the discharge frequency of individual units in responses to various stimuli (*cf* Bessou and Perl 1969 Torebjork and Hallin 1974 a)

Recording sites 6 recordings were made in sensory nerve fascicles in the peroneal nerve dorsolateral to the fibular head and 14 recordings in the superficial branch of the peroneal nerve (N Fibularis superficialis) 5—7 cm proximal to the ankle

Stimulation procedures

Electrical stimuli Uninsulated steel needles 0 3 mm in diameter served as stimulating electrodes and were inserted intradermally at interelectrode distances of 2—6 mm. A DISA stimulator unit (Type 14 E 01) delivered square wave pulses of 0 05—0 2 ms duration and amplitudes of 100—500 V at frequencies of 0 2—10 Hz

Mechanical stimuli Simple stimuli were used such as air puffs on the skin bending hairs slight touching and stroking with cotton wool or thin metal strings firm stroking with a small wooden stick pinching folds of the skin with a pair of forceps and pressing needles onto the skin Mapping of receptive fields and estimates of thresholds for mechanical stimuli were made with von Frey s hairs (Pressure Aesthesiometer Model PR 11 Research Media, Inc New York) An electromechanical transducer (contact area with skin 1 0 or 0 1 mm) was sometimes used to indicate the time course and strength of the stimuli

Thermal stimuli Cooling was achieved by ether evaporation from the skin Occasionally cooling was accomplished by running cold water through rubber tubes around the thigh and calf Radiant heat from an Infrared Spotheater (Philips Type TR 250/01) was used to warm the skin to temperatures perceived as warm or hot and noxious heat was achieved by briefly touching the skin with a glowing match

Skin temperature was measured by an Infrared Field Thermometer PRT 10L (Barnes Engineering Co Stanford Connecticut) or an IR Thermometer KT 41 (Heimann Wiesbaden Dotzheim West Germany)

Chemical stimuli Histamine liberation in the skin was induced by touching with nettle leafs (*Urtica urens*) Spicules of *Mucuna pruriens* (cowhage itch powder) were applied by gentle mechanical movements 1 drop of acetic acid (75 %) was applied on intact skin for up to 1 minute before removal 0 01—0 03 ml of potassium chloride (5 %) was injected intracutaneously

Receptive skin areas of afferent C fibres were first tested by gentle mechanical stimuli and moderate cooling and then high intensity stimuli were tried The stimuli were interrupted by test free intervals of 0 5—2 min in order not to depress the sensitivity of the nerve endings.

Pinpricking skin heating and application of chemicals were performed at the end of the experiments Deliberate tissue damage was not performed and the receptive endings were never made totally inexcitable by the stimuli

Reports of sensations evoked by the stimuli The subjects were instructed to report in their own words the sensations induced by various stimuli on the dorsum of the foot and ankle The loudspeaker sound of the neural activity was turned off when testing was performed and the subjects were generally prevented from hearing or seeing anything of the stimulation procedures

Material 46 afferent C units were analysed Their receptive fields were situated in skin areas sparsely covered with hairs on the dorsum of the foot and ankle except for three units with receptive fields in hairy skin on the lateral calf 16 units had potential amplitudes large enough (30—60 μV) to be identified by amplitude and shape in responses to various natural stimuli in the skin 30 units could not be individually identified in the original neurogram, since potentials of similar shape from several units with adjacent receptive fields interfered with each other Activity in these units was studied as latency changes in the C response to intradermal electrical stimulation when the units responded to natural stimuli in the skin (Hallin and Torebjork 1974 a) In addition five sympathetic units were classified by collision effects observed in the centripetally conducted C response to electrical intradermal stimulation (Hallin and Torebjork 1974 a) in association with manoeuvres known to increase the sympathetic outflow in human skin nerves (Hallin and Torebjork 1970 Hagbarth *et al* 1972 Delius *et al* 1972)

Results

Search for C fibre activity

A Orientation within the nerve and testing of electrode suitability for C fibre recording When an intraneural recording site was reached manoeuvres were performed which are known to induce massive, widespread sympathetic reflex responses in human cutaneous nerves (Hallin and Torebjork 1970 Hagbarth *et al* 1972 Delius *et al* 1972) If a sympathetic reflex was recorded in response for instance to a loud noise it was concluded that C fibre activity could be recorded from this electrode position since postganglionic sympathetic fibres are conducted at C velocity (*cf* Hagbarth and Vallbo 1968 Hallin and Torebjork 1974 b) In 70 recording sites where the sympathetic discharges were pronounced afferent C fibre activity could also be elicited from some part of the receptive field In those instances when no sympathetic activity was recorded tests for afferent C fibre activity were also negative If no sympathetic reflex activity could be recorded despite several adjustments of the electrode tip within the nerve the electrode was regarded as inadequate for C fibre recording and withdrawn from the nerve Inspection of the electrode tip in the microscope often disclosed loss of lacquer insulation for more than 100 μm at the tapered portion or a bent or blunt tip

B Testing for afferent C fibre activity Search for afferent C fibre activity was performed in two ways

1 The stimulation needles were inserted intradermally within the innervation zone of the impaled fascicle and the neural response to electric shocks of an intensity evoking a pricking pain sensation was checked for C fibre deflections Repeated adjustments of the stimulating electrodes to different parts of the skin were often necessary to find areas from where C fibre activity could be evoked If the latency of individual C fibre deflections in the electrically induced response increased when the electric shocks were combined with mechanical stimulation in defined skin areas

near the stimulating electrode and no latency changes or blockings occurred in association with manoeuvres known to increase the sympathetic outflow in skin nerves this was taken as evidence for afferent C fibre activation (Hallin and Torebjörk 1974 a)

2 Gentle mechanical stimuli and moderate cooling of the skin were made and if no response was obtained testing continued by firm strokings with a small wooden stick C fibre responses were identified by their long latency and they were often followed by afterdischarges in contrast to barrages of A fibre responses which ended abruptly after a stroke Conduction velocity determinations were then made with intradermal electrical stimulation This method was preferred since it allowed a quick testing for afferent C units responding to mechanical stimuli without a number of skin penetrations as with the first method

Consistent findings during these tests were that the A fibre responses to electrical intradermal stimulation were not as pronounced in the superficial peroneal nerve at the ankle as in the median nerve at wrist level and the C fibre responses to natural stimuli were generally not concealed to the same extent by concomitant A fibre activity as in the median and radial nerves (*cf* Torebjork and Hallin 1974 a) By contrast the C fibre activity was often distinct in the peroneal nerve and the identification of single C units was not exceptional Occasionally difficulties arouse in differentiating afferent C responses from sympathetic reflex responses at knee level where the latency of an afferent C response to stimulation in the big toe could be as long as 0 8 s (Fig 7) and the sympathetic reflex response induced by the same stimulus could appear at latencies which were only slightly longer (0 8—1 0 s)

Excitability changes in afferent C units during repeated electrical stimulation

If a combination of electrical and natural stimuli are used not only for identification of afferent C units but also for testing the receptive properties a crucial question is to what extent the excitability of the receptors to natural stimuli is influenced by the electrical stimulus itself For 6 of the C units the thresholds for activation by sustained pressure with von Frey s hairs were tested before and after a period of electrical stimulation A representative example is shown in Fig 1 This unit was activated by a pressure of 0 7 g (Fig 1 A) both before and during a period of electrical stimulation every 3 rd s (Fig 1 B) whereas stimuli of 0 4 g were ineffective in both instances The unit responses to various pressure stimuli could be directly observed in the neurogram and were also visualized as transient increases in latency of the electrically induced C unit responses in connection with application of the mechanical stimuli (Fig 1 C) indicating slowing of conduction velocity after period of increased firing (Hallin and Torebjörk 1974 a) On increasing the electrical stimulation frequency marked increases in latency were observed (Fig 1 D) corresponding to a decrease in peripheral conduction velocity in this fibre from about 0 55 m/s at 1 Hz to about 0 29 m/s after a short period of stimulation at 10 Hz when irregularities in latency and blockings appeared as an

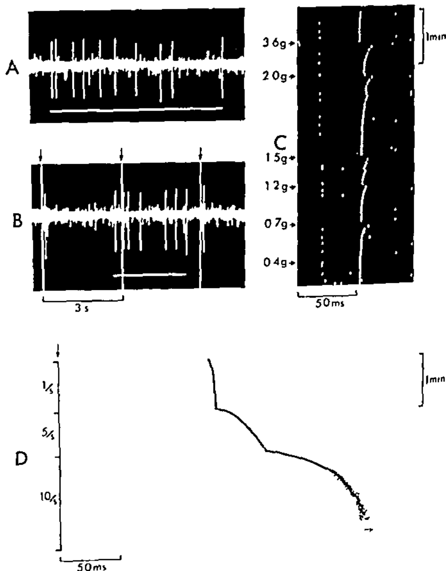


Fig 1 Responses in an afferent C unit recorded in th superficial peroneal nerve at the ankle Receptive field (1 × 1 mm) on the dorsum of the foot Conduction distan e 7 cm A Response to a pointed stimulus with on Frey's hair of 0 7 g (horizontal bar) before and B during a period of electrical intradermal stimulation every 3 rd s Electric shock artefacts indicated by arrows Time calibration identical in A and B C Successive C unit responses (dots) to electrical intradermal stimulation every 3 rd s and concomitant stimuli with von Frey s hairs (indicated to the left) Note transient increases in latency indicating slowing of conduction velocity associated with increased firing elicited by the natural stimuli First 75 ms after stimulus artefacts omitted D Increase in latency of C unit responses to electrical intradermal stimulation of constant intensity and various frequencies (indicated to the left) Figure retouched Stimulus artefacts indicated by arrow

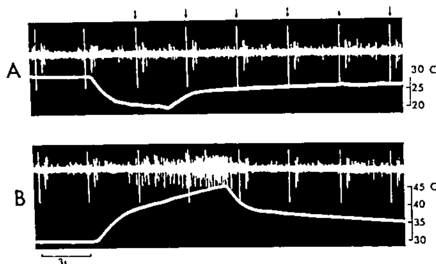


Fig 2 Multiunit C responses to electrical stimulation every 3 rd s (arrows) in the skin of the dorsum of the ankle Distance to recording site in the peroneal nerve at knee level 39 cm Lower traces indicate changes in temperature in the receptive skin field A Cooling the skin from 29 C to 20 C by ether evaporation did not induce any apparent C fibre activity B Increasing the skin temperature by radiant heat from 30 C to 45 C (perceived as heat) evoked a multifibre response starting at about 40 C and increasing with rise in temperature

indication of decreased excitability (*cf* Torebjork and Hallin 1974 b) 1 min after this stimulation period the threshold for mechanical stimuli with von Frey s hairs was still increased to about 2 0 g compared with about 0 7 g before the stimulation period

The results were similar for another four C units *i e* no obvious changes in mechanical threshold were detected with simple test methods after periods of low frequency electrical stimulation (0 2—0 3 Hz) but increased thresholds were regularly observed after periods of high frequency stimulation (5—10 Hz)

Long term electrical stimulation at 0 2—0 3 Hz usually induced minor changes in latency of the C responses In some cases a slight progressive increase was observed whereas other units showed a progressive decrease in latency probably due to increase in skin temperature in connection with reflex vasodilatation around the stimulating electrodes (*cf* Hallin and Torebjork 1973) Such changes in latency were possibly accompanied by changes in excitability at the receptor site as indicated in one C unit recording where skin reddening increase in skin temperature and sweat production around the stimulating electrodes were associated with an increase in conduction velocity of the C fibre The threshold for mechanical activation of the C unit decreased from 2 0 to 1 5 g and the reddened skin area was hyperaesthetic as compared with adjacent skin regions

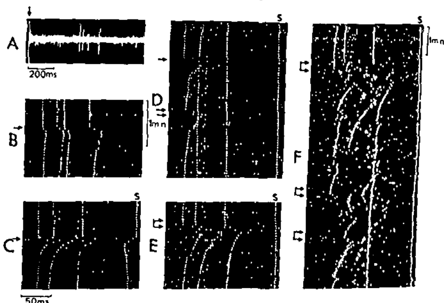


Fig 3 Same recording as in Fig 2 Vertical arrow indicates electric shock artefact Horizontal arrows indicate initiation of various natural stimuli during constant electrical stimulation every 3 rd s Time calibration in C identical in B—F where the first 375 ms after stimulus artefacts are omitted Calibration of analysis periods identical in B—E A At least 4 C units responded to a single electric stimulus in the receptive skin area B Cooling with ether (same sequence as in Fig 2 A) was accompanied by an increase in latency of all C components C Minute change in recording electrode position as compared with B One of the units in B had disappeared and a new unit s was recruited by the electrical shocks Radiant heat to 45 C (same sequence as in Fig 2 B) induced decrease in latency of one unit s and increase in latency of three other units D Selective activation of one C unit by repeated testing with a pointed stimulus of 2 g as indicated by changes in latency of the first C unit response E F Three units responded after application of itch powder (E) and repeated touching with nettle leafs on the skin (F) but the fourth unit s was not affected

Qualitative testing of 30 afferent C units in multi C fibre responses to electrical intradermal stimulation

Natural or electrical stimulation within small skin areas often induced responses in several C units indicating that the receptive fields were situated close to or overlapped each other If the potentials of several C units had similar amplitudes it was almost impossible to identify (in the original neurogram) activity from an individual unit among multifibre responses to a natural stimulus (Fig 2 B) The combination of electrical and natural stimuli was useful for a qualitative analysis of complex multi C unit responses This is illustrated in Fig 3 In this recording at least four different C units responded to intracutaneous electrical stimulation at 0 3 Hz on the dorsum of the foot (Fig 2 3 A) Cooling the skin from 99° C to 20° C by ether evaporation did not induce any afferent C unit discharges as indicated in Fig 2 A and it was concluded that the corresponding increase in latency of the C fibre responses to electrical stimulation (Fig 3 B) was due only to a temperature dependent decrease in conduction velocity (cf Franz and Iggo 1968) By contrast increasing the skin temperature by radiant heat from 30° C to 45° C evoked a

Fig 4 Examples of receptive field organization of C units recorded in the superficial peroneal nerve at the ankle The largest receptive field of a single C unit measured 0 6 × 1 7 cm and consisted of 7 receptive maxima (dots) surrounded by unresponsive areas The receptive fields of several C units encountered in *one* recording site were generally clustered together but occasionally as shown in the figure the receptive fields (schematically indicated as rings) of different units were separated by distances up to 7 cm Adjacent borders of the 4 receptive fields in the figure were separated by 0 3 5 and 2 cm



multifibre response (Fig 2 B) starting at about 40° C and increasing with rise in temperature This activity was accompanied by a transient *increase* in latency of the electrically induced responses in three C units and a *decrease* in latency for one C unit (Fig 3 C) The last unit indicated by s in Fig 3 was identified as sympathetic since collision effects were observed (but not illustrated) in association with a massive increase in sympathetic activity during periods of mental arithmetic (*cf* Hallin and Torebjork 1974 a) The sympathetic unit was probably not activated by skin heating and the decrease in latency was due to temperature dependent increase in conduction velocity The other three units were obviously activated by the skin heating since the temperature effect on conduction velocity was counter acted by a marked decrease in velocity as a consequence of repetitive firing

The first 3 C units in the electrically induced response could be individually activated by pointed stimuli with von Frey s hairs (Fig 3 D) allowing estimation of their receptive fields In this way complex receptive fields with 2—3 spot like receptive maxima separated by 1—3 mm were identified for 3 units whereas no area was found from where the sympathetic unit could be activated by pressure stimuli up to 15 g

Activity in the 3 afferent units was also induced after gentle application of cowhage spicules on the skin (Fig 3 E) and following repeated touching the skin with a nettle leaf (Fig 3 F) The sympathetic unit was not affected by these stimuli but served as a good control for constant experimental conditions with respect to intradermal electrical stimulation and recording site

In this way 30 afferent C units were identified Another 5 units were classified as sympathetic by collision tests They were generally not directly activated by natural stimuli in the skin but the possibility that intense stimuli induced not only reflex responses but also some antidromic impulses in sympathetic fibres could not be excluded Several additional C units were triggered by the electrical shocks but they could not be classified as afferent or sympathetic by the tests and manoeuvres used

The afferent C units were not spontaneously active at skin temperatures of 29—33 C and they were not activated by superficial touch stimuli by cotton wool bending hairs in the skin or moving a thin metal wire slowly over the skin mimicking the movements of an insect Pressure stimuli with von Frey s hairs of 0 7—8 5 g were necessary to evoke activity during concomitant electrical stimu

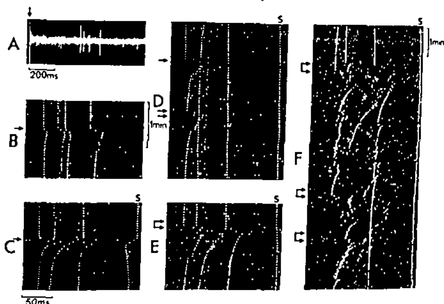


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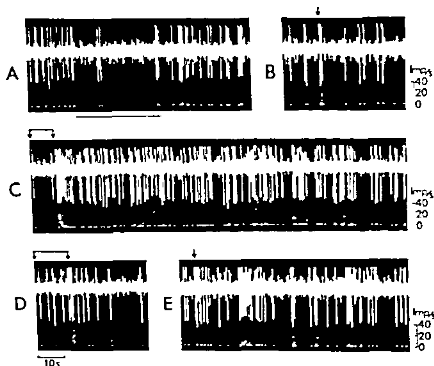


Fig 6 Activity in an afferent C unit recorded in the peroneal nerve at knee level Receptive area (3×3 mm) on the dorsum of the big toe Conduction distance 51 cm Conduction velocity 0 64 m/s Arrows mark initiation of various stimuli A The sustained activity at a skin temperature of 22 C was temporarily abolished by a transient increase in temperature to 25 C (bar) B Ether evaporation from the skin induced a brief burst of activity "Cold" C Application of a nettle leaf on the skin induced a burst of impulses ("itching pain") followed by irregular low frequency discharges ("itch") D Gentle application of itch powder also induced bursts of unit activity "Burning itch" E Irregular unitary bursts following application of a drop of acetic acid on the skin. "Pain and itch"

identified in response to natural stimuli without concomitant electrical stimulation A typical example is shown in Fig 5 As in the previous group of units these C units were not spontaneously active at normal skin temperature and they were not activated by light touching or moving stimuli The thresholds for activation with pointed mechanical stimuli of von Frey's hairs ranged 0 7—5.5 g and the receptive fields were often complex with several receptive maxima. The threshold for activation could be different among the receptive maxima of a unit Sustained pressure for several seconds was often necessary to evoke activity near threshold for activation whereas brief tests of similar intensities were ineffective A constant pressure with a nylon hair typically evoked a slowly adapting discharge (Fig 5 A) sometimes followed by an afterdischarge Stimuli of this type inducing responses at maximal instantaneous frequencies seldom exceeding 20 imp/s were often perceived at a

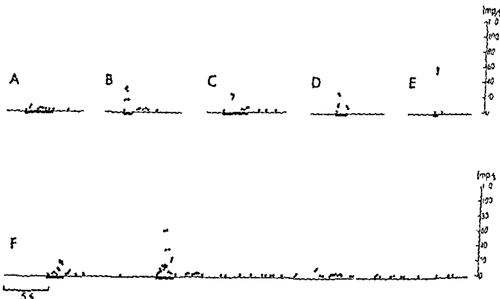


Fig. 5. Discharges of a polymodal C unit to mechanical, thermal and chemical stimuli in the skin of the dorsum of the foot. Conduction distance to the recording site in the superficial peroneal nerve at the ankle 8 cm. Conduction velocity 0.6 m/s. Instantaneous discharge frequency indicated to the right in Fig. 5—7. Thick horizontal bars indicate duration of various stimuli. Sensations reported by the subject are written within quotation marks in Figure texts 5—7. A. Slowly adapting response to a sustained pointed stimulus of ? g. "Itching". B. High frequency discharge elicited by a firm stroke, followed by afterdischarges of low frequency. "Smarting pain followed by nonpainful aftersensation". C. Response to squeezing the skin with a pair of forceps. "Pain". D. Response to needle prick. "Pricking and delayed pain". E. Intense response to briefly touching the skin with a glowing match. "Stinging and delayed burning pain". F. Intradermal insertion of injection cannula (left) followed after about 10 s by injection of 0.0? ml of 5 % KCl. Both procedures, especially injection of KCl, were painful. Note prolonged activity following KCl injection accompanied by reports of decreasing stinging sensation.

lation at low frequency (0.2—0.3 Hz). The receptive fields for pointed stimuli near threshold for activation were usually complex with 2 up to 7 spot like receptive maxima separated at 1—4 mm by less responsive areas, but occasionally only one receptive spot was found. The extensions of the receptive fields varied from less than 1×1 mm up to 6×17 mm. The receptive fields of units recorded in one fascicular site were generally situated close to each other, but occasionally the receptive fields of different units could be separated by up to 7 cm (Fig. 4).

All afferent units were activated by firm stroking of the skin, needle pricking and noxious heat. Application of itch powder and nettle leafs on the skin as well as intradermal injection of potassium chloride were also efficient stimuli for 23 units tested. Distal conduction velocities ranged 0.45—1.1 m/s.

Quantitative testing of receptive properties of 16 afferent C units

The number and frequency of discharges were analysed for another 16 afferent C units (with conduction velocities ranging 0.5—1.2 m/s) which could be individually

imp/s These stimuli were perceived as various intensities of 'pricking' or 'sharp pain' followed by prolonged stinging or burning pain Repeated intense heat stimulation known to induce sensitization of polymodal C nociceptors in the cat (Bessou and Perl 1969) was not performed

Chemical stimuli such as applying nettle leafs to the skin induced moderately intense bursts of impulses (Fig 6 C 7 H) accompanied by sensations of 'burning or itching pain' followed by irregular firing of lower frequency for several minutes associated with itching sensations Similar results were obtained after topical application of itch powder (Fig 6 D, 7 G) and inconsistently after application of acetic acid (Fig 6 E) Intense responses were induced by intradermal injection of potassium chloride (Fig 5 F) accompanied by burning pain and followed by a sustained low frequency discharge for up to 3 min when the subjects perceived a decreasing 'stinging' or 'aching' sensation

Two units were exceptional in the sense that they responded to cooling by ether evaporation in the skin at temperatures below 23° C One of these units (illustrated in Fig 6 and 7) was spontaneously active at a skin temperature of 22° C induced by running cold water through rubber tubes around the leg The irregular background discharge of this unit about 15—25 imp/min was first misinterpreted as activity in a sympathetic fibre some of which are spontaneously active at mean frequencies up to 15 imp/min (Hallin and Torebjörk 1974 b) However the discharge was not influenced by manoeuvres known to elicit sympathetic reflex responses and careful testing in the skin disclosed a small receptive skin area (3×3 mm) on the dorsum of the big toe The background firing was inhibited by increasing the skin temperature to 25 C (Fig 6 A) but reappeared again on skin cooling Ether evaporation induced a brief burst of activity (Fig 6 B)

Apart from the responses to cooling the two units exhibited similar polymodal receptive properties as the rest of the unit population They were not activated by touch stimuli and their mechanical thresholds were 1 2 and 3 6 g respectively They responded at instantaneous frequencies of 40—60 imp/s to intense mechanical heat and chemical stimuli

Discussion

In a previous study (Torebjork and Hallin 1974 a) only 8 afferent C units were studied in more than 200 recordings predominantly from the radial and median nerves at the wrist The probability to identify C fibre activity was improved in this investigation by a systematic search for C fibre impulses Manoeuvres inducing sympathetic reflex activity were useful to check the electrode suitability to record C fibre deflections and in those 20 recording sites where sympathetic activity was prominent afferent C fibre activity could also be recorded If this observation is further confirmed it should provide a simple and quick way for orientation towards recording sites in the fascicle where afferent C fibre activity can be identified The simultaneous recording of afferent and sympathetic impulses indicates that somatic -

and autonomic C fibres often are grouped together within the fascicles possibly enclosed within separate Schwann cell systems lying close to each other Direct anatomical evidence on this point is lacking due to difficulties in differentiating between somatic and autonomic unmyelinated axons from the electronmicroscopic picture (Ochoa and Mair 1969)

The C fibre activity was often distinct in the peroneal nerve and was generally not concealed by concomitant A fibre activity to the same extent as in the median and radial nerves These findings accord with a higher incidence of large myelinated fibres in nerves of the upper limb as compared with nerves in the lower limb (Sunderland *et al* 1949), as also indicated by higher conduction velocities in nerves of the upper extremity as compared with nerves in the leg (Buchthal and Rosenfalck 1966) According to light microscopy studies, the ratios of unmyelinated to myelinated fibres differ in different cutaneous nerves in man being relatively low in the median nerve at the wrist (Ranson *et al* 1935) Thus not only improved techniques in the search for afferent C fibre activity but also anatomic factors possibly account for the increased probability in recording C unit activity in the peroneal nerve

The experiments were designed to study the receptive properties of those afferent C units which were activated by low and moderately intense mechanical stimuli No attempts were made to identify C units specifically excited by small increases in skin temperature (Hensel *et al* 1960 Iruchijima and Zotterman 1960) Stimulation intensities strong enough to actually damage the skin were not used Hence specific thermoreceptors and very high threshold receptors were probably not recognized

The sequence of testing beginning with low intensity stimuli would favour the recognition of low threshold mechanoreceptors Since these receptors are known to fatigue on repeated stimulation (Iggo 1960 Bessou *et al* 1971) the stimuli were interrupted by test free intervals of 0 5—2 min in order not to depress the sensitivity of the nerve endings However touch stimuli mimicking the movements of an insect on the skin did not evoke any C fibre activity and cooling induced visible responses in only 2 units which had mechanical thresholds exceeding 1 g and responded most vigorously to intense mechanical heat and chemical stimuli Thus typical low threshold mechanoreceptive C units activated by mechanical stimuli of 0 01 to 0 045 g and cooling which constitute about 36 % of afferent C units in hairy skin supplied by the femoral cutaneous nerve in the cat (Bessou and Perl 1969) were not found in the present study The failure to record activity from low threshold mechanoreceptors has also been reported in previous microneurographic C unit recordings from the radial nerve innervating the dorsum of the hand (van Hees and Gybels 1972 Torebjork and Hallin 1974 a)

Could a bias in these recordings possibly in favour of detecting activity in the largest C fibres explain the failure to record impulse activity from low threshold C mechanoreceptors in man? According to occlusion experiments in the cat activity in C fibres responding to gentle mechanical stimuli (Douglas and Ritchie 1957) and mild cooling by 3—11° C (Douglas *et al* 1960) was found in the first part of

the C response derived from unmyelinated fibres of highest conduction velocity If there should be a similar correlation between conduction velocity of the unmyelinated afferent fibres and the responsiveness of their sensory terminals also in man a bias in the recordings favouring detection of activity in the largest C fibres is not likely to explain the failure to record impulses from low threshold C mechanoreceptors

In the cat these receptors are restricted to hairy skin and cannot be found in the hairless pad skin (Bessou *et al* 1971) The data so far obtained from human C unit recordings also indicate that they are few or absent in skin areas sparsely covered with hairs on the dorsum of the hand foot and ankle In future studies they should be looked for in skin regions with a rich supply of hairs The demonstration of their existence in man would be of considerable interest since activity in low threshold mechanoreceptors with A delta and C fibres has been associated with the sensation of tickling (Zotterman 1939)

The identification of afferent C units by combined electrical and natural stimuli has been discussed in a previous report (Hallin and Torebjörk 1974 a) This method proved to be useful in testing the receptive properties of groups of C units with adjacent or overlapping receptive fields and similar potential wave forms also in situations when identification of individual C elements was nearly impossible in the original neurogram (Fig 2 B) Even with electrical stimulation at low frequency changes in receptive properties of the units could not be excluded either as a consequence of the stimulation itself or indirectly by sudomotor (chemical) or vasomotor (volume and thermal) effects induced by stimulation Hence the C receptors were tested only *qualitatively* and not quantitatively by combined electrical and natural stimuli Furthermore the electric shocks tended to mask the sensations elicited by natural stimuli in the skin and hence no correlations between neural activity and sensation were performed during periods of combined stimuli With these restrictions in mind it is concluded that the C units tested by combined stimuli responded to a variety of mechanical thermal (heat) and chemical stimuli, thus exhibiting similar polymodal receptive characteristics as those 16 C units which could be tested more quantitatively

Several authors have identified C receptors with high thresholds for various types of stimuli in animal skin (Iggo 1959 Iriuchijima and Zotterman 1960 Witt 1962) and the term polymodal nociceptors was used for C units activated by moderately intense mechanical stimuli noxious heat and irritant chemicals (Bessou and Perl 1969) These polymodal nociceptors were not spontaneously active and did not respond to thermal changes in the range between 20 and 40° C Thresholds for punctate stimulators of the von Frey type was 0 2—45 g

The afferent C units in this study and also in previous C unit materials from the dorsum of the hand (van Hees and Gybels 1972 Torebjork and Hallin 1974 a) had similar polymodal receptive characteristics The most intense discharges were typically induced by intense stimuli threatening to damage the skin Like polymodal nociceptors in the cat human C receptors were effectively stimulated by scratching the

skin Such stimuli were often followed by afterdischarges but on repeated scratching at short intervals the responses as well as the afterdischarges sometimes decreased as an indication of fatigue These effects which are pronounced for low threshold C mechanoreceptors (Iggo 1960 Bessou *et al* 1971) may thus be traced also in other types of C receptors In contrast with polymodal nociceptors in the cat, the receptive fields were often complex with 2—4, occasionally 7 receptive maxima Polymodal nociceptors constitute about 30 % of afferent C fibres in hairy skin in the cat (Bessou and Perl 1969) and more than 80 % of afferent C fibres in the saphenous sural and median nerves in Rhesus monkeys (Kumazawa and Perl 1974) Similar receptors seem to be numerous also in the dorsum of the human hand foot and ankle

These C units respond rather non specifically to a variety of stimuli and the sensations aroused by various impulse patterns in these units are probably not related to the type of stimulus but rather to the intensity of stimulus It is realized that the stimuli used also evoked activity in A fibres and that the sensations were the results of integrative actions in the central nervous system being differently reported by various subjects Nevertheless, it is concluded that polymodal C receptors are active in responses to stimuli perceived as various intensities of burning or delayed pain and itch" Thus accords with a bulk of evidence suggesting that the delayed pain, heat sensation is associated with activity in C fibres (Zotterman 1933 Clark *et al* 1935 Zotterman 1939 Landau and Bishop 1953 Collins *et al* 1960 Torebjork and Hallin 1973) The sensation of itch has also been associated with C fibre activity both from differential blocking experiments (Bickford 1937) and latency measurements for itch perception from stimuli delivered to different sites of the body (Shelley and Arthur 1957) In analogy with first and second pain conveyed by fibres of different conduction velocities different types of itching sensations, described as pricking and burning have been reported (Chapman *et al* 1960) possibly indicating activation of different sets of fibres and receptors in production of itch sensations There are numerous points in the skin from where electrical mechanical thermal and chemical stimuli induce itching sensations and histological studies have shown that subepidermal areas of itch points are supplied with rich aggregates of unmyelinated nerve endings (Shelley and Arthur 1957) On more intense stimulation pain is perceived from these points and thus has led to the conclusion that both itch and painful sensations can be elicited from the same neural apparatus (von Frey 1922) The present results are not at variance with the idea that burning itch and delayed pain might be mediated by different impulse patterns from polymodal C receptors

Although not spontaneously active at normal skin temperature the C units could maintain an irregular or bursting discharge for several minutes after a change in the chemical milieu in the skin and a decrease in mechanical threshold was observed after a period of electrical stimulation producing evident vasomotor and sudomotor effects in the skin Decreased thresholds for various stimuli or sustained discharges could possibly be found in future studies of afferent C unit activity from human skin affected by painful or itching lesions

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References

BESSOU P and E R. PERL Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli *J Neurophysiol* 1969 *32* 1025—1043

BESSOU P P R. BURGESS E R PERL and C B TAYLOR Dynamic properties of mechanoreceptors with unmyelinated (C) fibers *J Neurophysiol* 1971 *34* 116—131

BICKFORD R G Experiments relating to the itch sensation its peripheral mechanim and central pathways *Clin Sci* 1937 *3* 377—386

BUCHTHAL F and A ROSENFALCK Evoked acion potentials and conduction velocity in human sensory nerves *Brain Res* 1966 *3* 1—122

CHAPMAN L F H GOODELL and H G WOLFF Structures and processes involved in the sensation of itch In *Advances in Biology of Skin Vol I Cutaneous Innervation* (Ed W Montagna, Pergamon Press Oxford) 1960 161—188

CLARK D J HUGHES and H S GASSER Afferent function in the group of nerve fibres of slowest conduction velocity *Amer J Physiol* 1935 *114* 69—76

COLLINS W F F E. NULSEN and C T RANDT Relation of peripheral nerve fiber size and sensation in man *Arch Neurol* 1960 *3* 381—385

DELIUS W K. E HAGBARTH A HONGELL and B G WALLIN Manoeuvres affecting sym pathetic outflow in human skin nerves *Acta physiol scand* 1972 *84* 177—186

DOUGLAS W W and J M RITCHIE Non medullated fibres in the saphenous nerve which signal touch *J Physiol* (Lond) 1957 *139* 385—399

DOUGLAS W W J M RITCHIE and R. W STRAUB The role of non myelinated fibres in signalling cooling of the skin *J Physiol* (Lond) 1960 *150* 266—283

FRANZ D N and A. IGGO Conduction failure in myelinated and non myelinated axons at low temperatures *J Physiol* (Lond) 1968 *199* 319—345

VON FREY M Zur physiologie der Juckempfindung *Arch neerl Physiol* 1922 *7* 142—145

HAGBARTH K E and A B VALLBO Pulse and respiratory grouping of sympathetic impulses in human muscle nerves *Acta physiol scand* 1968 *74* 96—108

HAGBARTH K E A HONGELL R G HALLIN and H E TOREBJORK Afferent impulses in med an nerve fascicles evoked by tactile stimuli of the human hand *Brain Res* 1970 *24* 423—442

HAGBARTH K E R G HALLIN A HONGELL H E TOREBJORK and B G WALLIN General characteristics of sympathetic activity in human skin nerves *Acta physiol scand* 1972 *84* 164—176

HALLIN R G and H E TOREBJORK Afferent and efferent C units recorded from human skin nerves in situ *Acta Soc Med upsalien* 1970 *75* 277—281

HALLIN R. G and H E TOREBJORK Electrically induced A and C fibre responses in intact human skin nerves *Exp Brain Res* 1973 *16* 309—320

HALLIN R. G and H E TOREBJORK Single unit sympathetic activity in human skin nerves during rest and various manoeuvres *Acta physiol scand* 1974 b *92* 303—317

HALLIN R. G and H E TOREBJORK Methods to differentiate electrically induced afferent and sympathetic C unit responses in human cutaneous nerves *Acta physiol scand* 1974 a *92* 318—331

VAN HEES J and J M GYBELS Pain related to single afferent C fibers from human skin *Brain Res* 1972 *48* 397—400

HENSEL H A IGGO and I WITT A quantitative study of sensitive cutaneous thermoreceptors with C afferent fibres *J Physiol* (Lond) 1960 *153* 113—126

IGGO A Cutaneous heat and cold receptors with slowly conducting (C) afferent fibres *Quart J exp Physiol* 1959 *44* 362—370

IGGO A Cutaneous mechanoreceptors with afferent C fibres *J Physiol* (Lond) 1960 *152* 337—353

IRIUCHIJIMA J and Y ZOTTERMAN The specificity of afferent cutaneous C fibres in mammals *Acta physiol scand* 1960 *49* 267—278

KUMAZAWA T and E R. PERL Characteristics of cutaneous sensory units with unmyelinated (C) fibers in the monkey *J Neurophysiol* 1974 In press

LANDAU W and G H BISHOP Pain from dermal periosteal and fascial endings and from inflammation *Arch Neurol Psychiat* (Chic) 1953 *69* 490—504

OCHOA J and W G MAIR The normal sural nerve in man I Ultrastructure and numbers of fibers and cells *Acta neuropath (Berl) 1969 13 197—216*

RANSON S W W H DROEGEMUELLER H K DAVENPORT and C FISCHER Number size and myelination of the sensory fibers in the cerebrospinal nerves In *Sensation Its mechanisms and disturbances Ass Res Nerv Ment Dis* 1935 *15* 3—34

SHELLEY W B and R P ARTHUR The neurohistology and neurophysiology of the itch sensation in man *A M A Arch Dermatol* 1957 *76* 296—323

SUNDERLAND S J O LAVARACK and L J RAY The caliber of nerve fibers in human cutaneous nerves *J comp Neurol* 1949 *91* 87—101

TOREBJORK H E and R G HALLIN C fibre units recorded from human sensory nerve fascicles in situ *Acta Soc Med upsalien* 1970 *75* 81—84

TOREBJORK H E and R G HALLIN Perceptual changes accompanying controlled preferential blocking of A and C fibre responses in intact human skin nerves *Exp Brain Res 1973 16* 321—332

TOREBJÖRK H E and R G HALLIN Identification of afferent C units in intact human skin nerves *Brain Res* 1974 a *67* 387—403

TOREBJORK H E and R G HALLIN Responses in human A and C fibres of repeated electrical intradermal stimulation *J Neurol Neurosurg Psychiat 1974* b *37* 653—664

VALLBO A B and K E HAGBARTH Activity from skin mechanoreceptors recorded percutaneously in awake human subjects *Exp Neurol* 1968 *21* 270—289

WITT I Aktivitat einzelner C Fasern bei schmerzhaften und nicht schmerzhaften Hautreizen *Acta neuroveget* (Wien) 1962 *24* 208—219

ZOTTERMAN Y *Studies in the peripheral nervous mechanism of pain Acta med scand* 1933 *80* 185—242

ZOTTERMAN Y Touch pain and tickling an electrophysiological investigation on cutaneous sensory nerves *J Physiol* (Lond) *1939 95* 1—28

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Acta physiol scand 1974 92 391—398

From the Department of Surgical Gastroenterology C Rigshospitalet and Department of Clinical Chemistry Bispebjerg Hospital Copenhagen Denmark

Effect of Atropine on Hypoglycemic Release of Gastrin in Man

By

F Stadil J Malmstrom J F Rehfeld and M Miyata

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Abstract

Stadil F J Malmstrom J F Rehfeld and M Miyata *Effect of atropine on hypoglycemic release of gastrin in man* Acta physiol scand 1974 92 391—398

Hypoglycemic release of gastrin has been supposed solely to be due to vagal release In this report the atropine resistance of the gastrin response to insulin was examined 9 normal subjects were studied twice 0 2 IU of insulin/kg i v was injected in each study but in one atropine (30 μg/kg i m) was injected prior to insul n Serum gastrin concentrations were measured by radioimmunoassay Blood glucose concentrations did not differ in the two studies Atropine almost abolished gastric output of acid Gastrin concentrations rose after insulin and slightly more after atropinization In four of the subjects a second late gastrin increase was observed after atropine It is concluded that in man a major part of the hypoglycemic release of gastrin is independent on a cholinergic mechanism

Cholinergic release of gastrin has been supposed to be the final common path for all stimuli capable of releasing gastrin The basis for this hypothesis is studies in dogs with indirect assessment of gastrin release (Elwin and Uvnas 1966 Schofield 1966 Grosman 1967)

However in the dog antral denervation abolishes the gastrin response to insulin (Csendes Walsh and Grossman 1972) whereas the release of gastrin after insulin is only slightly influenced by vagotomy in man (Stadil 1974)

This investigation was undertaken to evaluate whether gastrin response to insulin in man is atropine resistant or not

Methods

Subjects 9 healthy medical students 7 males and 2 females aged from 19 to 25 years we e tud ed

Int t gat ve procedure After an overnight fast a rad o-opaque nasogastric tube was nserted tl e posit on was controlled by fluoroscopy After emptying the stomach gastr c juice was aspirated continuously by intermittent pump suction Sixteen 15 min collections were made

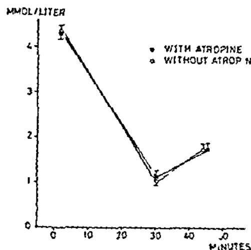


Fig 1 Blood glucose concentrations before and after insulin 0 2 IU/kg i.v in 9 normal subjects Black symbol indicate prior injection of atropine sulphate (30 µg/kg i.m)

4 before and 12 after injection of 0 2 IU of insulin per kg b w t i.v All subjects were studied twice with and without atropine sulphate (30 µg/kg i.m) in the course of a few days Atropine injection preceded insulin injection by 25 min Blood was drawn from the cubital vein through an indwelling cannula before and at 15-min intervals throughout the investigation Serum was stored at −20 C until assay

Laboratory analysis Gastric acid was measured using an Autotitrator TTT (Radiometer Copenhagen) Titration end point was pH 3 5 Glucose was determined by the oxidase method Gastrin was measured by radioimmunoassay (Stadil and Rehfeld 1973) using antiserum 2604 7 (Rehfeld Stadil and Rubin 1972) With this antiserum sulphated and non-sulphated forms of gastrin 13 gastrin 17 gastrin 34 and the gastrin component I (Rehfeld 1973 Rehfeld Stadil and Vikelsøe 1974) are measured Synthetic human gastrin I (SHG) from Imperial Chemical Industries Macclesfield England was used as standard and purified monoiodinated I labelled SHG (Stadil and Rehfeld 1972) as tracer Final dilution of unknown samples in the assay was 1 10 Details on the assay were reported earlier (Stadil and Rehfeld 1973) All gastrin determinations were done within one month all samples from one subject were assayed simultaneously

Calculations Results were evaluated statistically by the Wilcoxon test for paired differences Peak gastrin concentration was taken to be the mean of the highest 3 consecutive gastrin concentrations after insulin Basal gastrin concentration as the mean of the 2 last samples before insulin injection

Results

Blood glucose concentrations appear from Fig 1 No difference was observed whether atropine was given or not Glucose concentrations in plasma always decreased below 1 6 mmol per liter

Acid concentrations and acid output in the gastric juice are shown in Fig 2 Atropinization caused a reduction of the peak concentration to about 40 % and almost abolished acid output in response to insulin The inhibition was maintained throughout the investigation

Mean gastrin concentrations are shown in Fig 3 Basal levels of gastrin before insulin were not affected by atropine (Table I) Without atropine gastrin concentrations increased to a maximum within the first hour after insulin Mean con

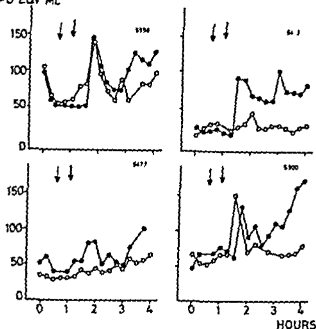


Fig 4 Individual curves showing serum gastrin concentrations (see Fig 2) in the 4 subjects demonstrating a late increase in gastrin concentrations after insulin

subjects (Fig 4) When peak concentrations with and without atropine were compared the values after atropine were significantly higher (Table I) When changes were studied only in the first hour after insulin differences were insignificant (Table I) The gastrin response to insulin varied markedly in different individuals 4 of the subjects responded poorly both with and without atropine

Discussion

Our study demonstrates that in man atropine does not decrease the basal concentrations of gastrin in serum Furthermore the gastrin response to hypoglycemia was not diminished by the dose of atropine used The findings suggest that insulin hypoglycemia induces release of gastrin independent of cholinergic excitation

The ability of atropine to influence cholinergic effects in different organs differs The dose of atropine used in this study was quite large in the respect that it exceed the dose usually recommended to be given to man Admittedly though it may have been too small to prevent cholinergic release of gastrin On the other hand the dose of atropine did suppress gastric activation by insulin when assessed by acid secretion (Fig 2)

Hypothetically cholinergic release of gastrin may be less important in man than has been presumed The concept that gastrin release is cholinergic resulted from studies in dogs where release of gastrin from the antrum was assessed by stimulation of acid secretion in denervated pouches of the oxyntic area In these dogs

TABLE II Summary of studies on the effect of injection of atropine on basal concentrations of serum gastrin in dog and man

Authors	Dose of Atropine µg/kg b w	Species and number of subjects	Gastrin concentrations
Nilsson Simon and coworkers *1972*	63 i v	Dogs (4)	No change
Csendes Walsh and Grossman 1972	100 subc	Dogs (4)	Increase
Debas Walsh and Grossman 1973	200 subc	Dogs (6)	Decrease
Reeder Jackson and coworkers *1970*	9 i v	Man 1 normal 3 d ulc	Increase
Walsh Yalow and Berson 1971	14 i m	Man 5 normals 3 d u 1 p a	 No change No change No change
Ganguli and Hunter 1972	11 i m	Man 3 normals	Decrease
Hansky and Korman 1973	9 i m	Man 22 normals 20 d u 10 g u	 No change No change No change
Schrumpf and Myren 1974	10 i v	Man 5 normals 9 ach.	 No change Decrease
Farooq and Walsh 1974	15 m	Man 5 normals	No change

Abbreviations d u = duodenal ulcer g u = gastric ulcer ach = achlorhydria p a = pernicious anemia

the only gastrin releasing agent not suppressed by local anesthesia is acetylcholine and this agent in turn can be rendered impotent by topical atropine (Elwin and Uvnas 1966 Schoefield 1966 Grossman 1967) The presence of important species differences has long been recognized as Blair (1967) reported that gastrin release by a feeding stimulus was not suppressed by atropine in the anesthetized cat

Atropine and basal gastrin levels After development of radioimmunoassays for gastrin a number of studies have reported on the effect of systemic atropine on serum gastrin concentrations in man and dog Table II summarizes the results recorded in the literature Even if discrepancies are apparent the main impression is that atropine does not change the fasting level which was also found in this investigation Hence it seems that basal gastrin level is not maintained by a tonic cholinergic release A notable exception may be patients with achlorhydria (Schrumpf and Myren 1974)

TABLE III Summary of studies on the effect of atropine on the gastrin response to various stimuli in dogs

Authors	Dose of Atropine µg/kg	Stimulus	Subjects and (N)	Gastrin Response
Csendes Walsh and Grossman 1972		Insulin 0 5 IU/kg	Innervated A and P pouches	
	100 s c	i v	(4)	Abolished (2)
	200 s c	—	(2)	Abolished (2)
	150 s c	Feeding	P pouch (2)	No chang
Nilsson Simon and coworkers 1972	70 – 100 i v	Sham feeding	P pouch (2)	Abolished
	90 – 100 i v	Feeding	P Pouch (2)	No change
Debas Walsh and Grossman 1973	200 s c	Acetylcholine Bovril Distention 2 Deoxyglucose	Innervated A and H pouches (6)	Abolished and decrease below basal

A P and H pouches mean Antral Pavlov and Heidenhain pouches

Atropine and stimulated gastrin release in the dog According to the few earlier reports the gastrin response to insulin (Csendes, Walsh and Grossman 1972) sham feeding (Nilsson et al 1972) 2 deoxyglucose, distension and bovril (Debas Walsh and Grossman 1973) is abolished by atropine It is not quite clear how gastrin responses to feeding are affected (Table III) With the possible exception of feeding responses the studies confirm that release of immunoreactive gastrin mainly depends on cholinergic mechanisms in the dog

Atropine and stimulated gastrin release in man In contrast with the findings in dogs vagotomy does not abolish the hypoglycemic release of gastrin in man (Stadil 1972 Stadil and Rehfeld 1974) which demonstrates that in man at least part of the gastrin response to insulin is non vagal The present study shows that a major part of the response is non cholinergic as well

Two earlier investigations dealt with atropine and gastrin responses to feeding in man (Walsh Yalow and Berson 1971 Hansky and Korman 1973) Both reported an unchanged or increased release of gastrin after atropinization (9 to 15 µg/kg b w) Preliminary reports on the effect of atropine on insulin stimulation agree with the findings of the present study Farooq and Walsh (1974) found an increased peak gastrin response to insulin hypoglycemia after atropinization when intragastric pH was maintained at 5 5 throughout by instillation of bicarbonate In patients with duodenal ulcer Schrumpf (1974) found a considerably higher gastrin response to insulin and atropine than to insulin without atropine Hence at present little evidence is favouring the idea of cholinergic gastrin release in the human subject

In this study gastric juice was aspirated continuously to reduce differences in acid inhibition of gastrin release Complete diversion was probably never obtained However the effect of insulin combined with atropine could not be due to an in direct action via acid suppression because atropine did not change basal levels of gastrin significantly and the increase in gastrin concentrations did not parallel the decrease in acid secretion An alternative speculation is that in man gastrin release is suppressed by inhibitory agents secreted by a tonic cholinergic mechanism This might explain unexpected actions of atropine

In previous studies in human subjects peak gastrin concentrations were found 45 or 60 min after insulin and even if gastrin concentrations often remained elevated no late increase was noted In the rat peak concentrations occur 240—270 min after insulin (Hakanson *et al* 1974) To our knowledge gastrin was only measured for 90 to 120 min after insulin in all previous studies in humans (Stadil 1974) In this report a late increase starting 120 min after insulin was observed This late increase was only seen in 4 of the subjects (Fig 4) and was more evident in the studies with atropine We have no satisfactory explanation of this but a late acid response to insulin is observed in some subjects (Porter Longmire and French 1953 Sun and Shay 1960) This late response might possibly be mediated by gastrin

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References

Blair E L The effect of tissue extracts on the stomach *Gastric secretion mechanisms a d cont ol* Editors *T K Schnitka I A L Gilbert and R C Harrison* Pergamon Press Oxford 1967 263—281

Csendes A J H Walsh and M I Grossman Effects of atropine and of antral acidification on gastrin release and acid secretion in response to insulin and feeding in dogs *Gastro nterology* 1972 *63* 257—263

Debas H T J H Walsh and M I Grossman Search for atropine resistant mechanism of gastrin release in dog *Gastroente ology* 1973 *64* 856

Elwin C E and B Uvnas Distribution and local release of gastrin *Gast in* Editor M I Grossman Butterworths, London 1966 69—82

Farooq O and J H Walsh Atrop ne increases serum gastrin response to insulin in man In press

Grossman M I Neural and hormonal stimulation of ac d secret on *Handbook of Physiology* section 6 volume II Editor C F Code American Physiological Society Washington DC 1967 835—863

Håkanson R G Liedberg J Oscarson J F Rehfeld F Stadil and J H. Kroeler Regulation of rat tomach h stid ne decarboxyglase activity by gastrin In p ess

Hansky J and M G Korman Immunoassay studies in pept c ulcer *Clin Gastroenterology* 1973 2 275—291

Nilsson G J Simon R S Yalow and S A. Berson Plasma gastrin and gastric acid response to sham feeding and feeding in dogs *Gast oenterology* 197? *63* 51—59

Porter R W R L Longmire and J D French Neurohumeral influence on gastric hydrochloric acid secretion *F d Proc* 1953 *12* 110

Rehfeld J F Gastrins n serum A re iew of gastrin radioimmunoanalysis and the discovery of gastrin heterogene t n serum *Scand J Gast oent* 1973 *8* 577—583

Rehfeld J F F Stadil and B Rubin I roduction and evaluation of antibodies to ga trin for radioimmunoassay *S and J cl n Lab Invest* 1972 *30* 2?1—23?

REHFELD J F F STADIL and J VIKELSOE Immunoreactive gastrin components in human serum *Gut* 1974 *15* 102—111
SCHOFIELD B Inhibition by acid of gastrin release *Gastrin* Editor M I Grossman Butterworths London 1966 171—192
SCHRUMPF E Personal communication
SCHRUMPF E and J MYREN The effect of atropine on the plasma concentration of gastrin in fasting subjects *Scand J Gastroent* 1974 *9* 123—125
STADIL F Effect of vagotomy on gastrin release during insulin hypoglycaemia in ulcer patients *Scand J Gastroent* 1972 *7* 225—231
STADIL F Gastrin and insulin hypoglycaemia A review of studies on gastrin determination and hypoglycaemic release of gastrin in man *Scand J Gastroent* 1974 9 supp 23 1—46
STADIL F and J F REHFELD Preparation of ^{125}I labelled synthetic human gastrin I for radioimmunoassay *Scand J clin Lab Invest* 1972 *30* 361—368
STADIL F and J F REHFELD Determination of gastrin in serum An evaluation of the reliability of a radioimmunoassay *Scand J Gastroent* 1973 *8* 101—117
STADIL F and J F REHFELD Gastrin response to insulin after selective highly selective and truncal vagotomy *Gastroenterology* 1974 *66* 7—15
SUN D C H and H SHAY Mechanism of action of insulin hypoglycaemia on gastric secretion in man *J appl Physiol* 1960 *15* 697—703
WALSH J H R S YALOW and S A BERSON The effect of atropine on plasma gastrin response to feeding *Gastroenterology* 1971 *60* 16—21

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From the Department of Experimental Medicine Pharmacia AB Uppsala and the Department of Pharmacology Karolinska Institutet Stockholm Sweden

Microvascular Flow Velocity in Cat Omental Adipose Tissue as Affected by Sympathetic Nerve Stimulation

By

SUNE ROSELL MARCOS INTAGLIETTA [1] RONALD F TUMA and KARL E ARFORS

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Abstract

ROSELL S M INTAGIETTA R F TUMA and K E ARFORS *Microvascular flow velocity in cat omental adipose tissue as affected by sympathetic nerve stimulation* Acta physiol scand 1974 92 399—403

The effect of sympathetic nerve stimulation on the microvascular red blood cell flow velocity was measured in cat omental adipose tissue Electrical impulses of 10 V 0 5—6 Hz were utilized to stimulate the appropriate branch of the splenic nerve Microvascular velocities were measured in the transilluminated omental bursa in arterioles capillaries and venules on line by means of a dual window television system The responses noted were 1) decrease in flow (75 % of vessels) 2) no response (7 %) and 3) increase (18 %) The magnitude of the responses were in proportion to the stimulatory frequency The disparity of responses may be related to previous morphologic observations on the non uniformity of innervation of canine subcutaneous adipose tissue It is proposed that the various vascular reactions reflect the presence of compartments in the same tissue endowed with different sympathetic controls

Indexable words Sympathetic activity adipose tissue capillary flow lipolysis television microscopy

By using the fluorescence method of Falck and Hillarp to visualise adrenergic nerve fibres in white adipose tissue (subcutis mesentery and omentum) a rich peri vascular plexus of fluorescent varicose nerves was found around arteries and veins (Diculescu and Stoica 1970 Ballard Malmfors and Rosell 1974) Nerve fibres could also be found between adipocytes In adjacent areas however no adrenergic nerve fibres were identified *This indicated that there are two compartments in adipose* tissues from the same region one which is innervated by adrenergic fibres and an other which is not (Ballard Malmfors and Rosell 1974) This in turn may suggest that the vascular control and the lipolysis is governed by differen mechanisms in various compartments of adipose tissue In order to further elucidate this possibility the microcirculation of omental tissue of the cat has been studied by means of quantitative intravital techniques

[1] Present address AMES Bioengineering University of California San Diego La Jolla California 92037 U S A

Results

Experiments were performed on 9 different experimental animals and flow record were obtained from a total of 80 different arterioles, capillaries and venules in clear tissue adjacent to adipose cells

3 different types of responses were noted 1) a decrease in flow (75 % of the vessels) 2) no response (7 %) 3) an increase in flow (18 %) Both increases and decreases in flow velocities were graded i e the response augmented in magnitude as the frequency of stimulation was increased (Fig 1 and 2) Visual inspection of the vessels and surrounding tissue which showed opposing reactions did not reveal any basic morphologic or functional differences It was also seen that decreases in flow resulted from constriction of small arteries of approximately 40—50 μm in diameter No comparable effect was noted for the cases where flow increased

In vessels where the flow decreased as a result of stimulation (Fig 1) an initial increase in flow velocity which amounted to 0 05—0 1 mm/s was frequently noted Subsequently the flow decreased and in some instances stopped althogether depending on the frequency utilized (Fig 1) In a few occasions stimulatory escape occurred i e the flow increased gradually after reaching a low value while the stimulatory signals were applied Reactive hyperaemia was absent in those vessels which showed decreased flow After stimulation flow velocity gradually returned to the pre stimulaory level The time required for the flow to return to the control value was approximately 3—5 min No basic differences were found between the responses of arterioles capillaries and venules in a given microvascular network supplied by the same small artery

Vessels and microvascular networks which exhibited an increase in flow as a consequence of stimulation (Fig 2) returned to control values somewhat more slowly than in those showing the opposite effects Adjacent areas supplied from different small arteries often exhibited the two types of opposite reactions as shown in Fig 3 The decrease in flow velocity could be inhibited by the administration of phenoxybenzamine 10 mg/kg intravenously (Fig 4)

Discussion

The results obtained can be considered to exemplify the flow behaviour throughout the microvasculature of the omentum When a given type of effect was noted as a result of nerve stimulation i e increase in flow velocity no effect or decrease the whole vascular network supplied by one reacting small artery showed the same qualitative change irrespective of resting flow velocity Sympathetic nerve stimulation resulted in vasomotor reactions (constriction or dilatation) in more than 90 % of the vessels measured This indicates that most of the resistance vessels are innervated by sympathetic nerves However in some areas no reaction was noted This may indicate an unequal distribution of innervation of the microvasculature in adipose tissue

The analysis of the morphology of canine adipose tissue performed by Ballard Malmfors and Rosell (1974) where the adrenergic innervation was put in evidence by means of the fluorescense technique of Falck and Hillarp (see Malmfors 1965) revealed that the adrenergic nerves could be traced to discrete circumscribed areas, whereas adjacent areas seemed to lack adrenergic innervation It could not be excluded however that this finding was due to technical and methodological difficulties which prevented the proper identification of all adrenergic nerve fibres Our present findings with a different technique support the hypothesis of different compartments as far as adrenergic innervation is concerned

The vascular reactions which cause the observed variety of responses needs further elucidation In particular the observed increases in flow could be due to two different mechanisms On one hand the noted difference in innervation could lead to an increase in vascular resistance in inervated areas which could cause an increase in precapillary pressure and thus increase the flow in other areas Secondly the same phenomenon could also be the result of an uneven distribution of α and β receptors which could cause simultaneous vasodilation and vasoconstriction in the same tissue in accordance to the local proportion of these two receptors

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References

ARFORS K E D BERGQVIST M INTAGLIETTA and B WESTERGREN Measurement of blood flow velocity in the microcirculation *Uppsala J Med* In press

BALLARD K and S ROSELL Adrenergic neurohumoral influences on circulation and lipolysis in canine omental adipose tissue *Circulat Res* 1971 *28* 389—395

BALLARD K T MALMFORS and S ROSELL Adrenergic innervation and vascular patterns in canine adipose tissue *Microvasc Res* 1974 In press

DICULESCU I and M STOICA Fluorescence histochemical investigation on the adrenergic innervation of the white adipose tissue in the rat. *J Neurovisc Relat* 1970 32 25—36

INTAGLIETTA M and W R TOMPKINS System for the measurement of velocity of microscopic particles in liquids *IEEE Trans Biomed Eng* 1971 *18* 376—377

INTAGLIETTA M W R TOMPKINS and D R RICHARDSON Velocity measurements in the microvasculature of the cat omentum by on line method *Microvasc Res* 1970 2 462—473

MALMFORS T Studies on adrenergic nerves *Acta physiol scand* 1965 *48* 1—93

WAYLAND H and P C JOHNSON Erythrocyte velocity measurements in microvessels by a two slit photometric method *J appl Physiol* 1967 22 333—337

Acta physiol scand 1974 92 404—411

From the Department of Zoophysiology University of Goteborg Sweden

Potentiation and Inhibition of Noradrenaline Induced Contractions of the Rat Portal Vein in Anion Substituted Solutions

By

Bo A Wahlstrom and Bo Svennerholm

Received 6 May 1974

Abstract

Wahlstrom B A and B Svennerholm *Potentiation and inhibition of noradrenaline induced contractions of the rat portal vein in anion substituted solutions* Acta physiol scand 1974 92 404—411

The effect of anion substitution on spontaneous mechanical activity and noradrenaline induced excitation of the rat portal vein was studied. When chloride was substituted by nitrate and bromide the spontaneous activity was increased while pyruvate isethionate and benzenesulphonate decreased it. When noradrenaline was given initially the response was potentiated in all solutions The order of potency was benzenesulphonate > isethionate > pyruvate ⩾ Br ⩾ NO_3 When dose response curves for noradrenaline were made in normal solution and in anion-substituted solutions it was found that NO_3 and Br increased the sensitivity of the muscle cells to noradrenaline while in isethionate and benzenesulphonate solutions the responsiveness was reduced Stepwise substitution of Cl by NO_3 or Br showed that the effect of noradrenaline was inversely related to [Cl] When the membrane was depolarized by solutions with excess $[K]_o$ the response to noradrenaline gradually fell although a response was still present in high K-solution It is concluded from the experiments that the distribution and permeability of anions greatly influence the excitatory effect of noradrenaline on the rat portal vein

Noradrenaline acts on venous smooth muscle by depolarizing the membrane and eliciting a large contractile response (Axelsson *et al* 1966 It has been proposed that the depolarization caused by the drug is due to an increased chloride permeability (Wahlstrom 1973 a Since chloride seemed to be involved in the noradrenaline action in this preparation the response to the drug was tested in solutions with varying Cl_o Cl ratios and or different membrane potentials In this way a relationship between the noradrenaline induced contraction and membrane potential and E_{Cl} might be established

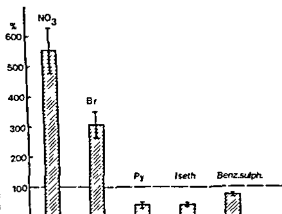


Fig 1 The effect of anion substitution on spontaneous activity The values are expressed as percent of the activity in normal PSS Each bar is shown with ± 2 S E (n = 8)

Methods

Male Sprague Dawley rats weighing 250—300 g were used They were stunned by a blow over the neck and bled out The portal vein was rapidly dissected mounted in an organ bath of 50 ml and connected to a force-displacement transducer for recording of mechanical activity Muscles were allwoed 1 h recovery in normal physiological salt solution (PSS) before being subjected to drug administration or anion substitution The mechanical activity was recorded on a Grass Polygraph and continuously integrated by means of an electronic integrator All results were calculated as mean tension per min

The normal physiological salt solution (PSS) made up from isotonic stock solutions had the following composition Na^{+} 137 5 K^{+} 6 0 Ca^{++} 2 5 Mg^{++} 1 2 Cl^{-} 134 1 HCO_3^{-} 15 5 $H_2PO_4^{-}$ 1 2 and glucose 11 5 mM All solutions were continuously bubbled with a gas mixture containing 97 % O_2/3 % CO_2 and pH was 7 4 In the substitution experiment Cl^{-} was substituted by NO_3^{-} Br^{-} CH_3COCOO^{-} (pyruvate) $CH_3CH_2SO_3^{-}$ (Isethionate) or $C_6H_5SO_3^{-}$ (benzenesulphonate) on an equimolar basis The Cl^{-} concentration in the substituted solutions was 13 mM unless otherwise stated Noradrenaline was obtained as pure substance (Sigma) and dissolved in 10 ml of bidistilled water to which has been added a drop of 1 M HCl Further dilution were made as required in the various salt solutions All concentrations of noradrenaline are given in μM Numerical values of drug effects are given as mean ± S E and differences between means considered significant if $p \leqslant 0.05$ as determined by Student's t test

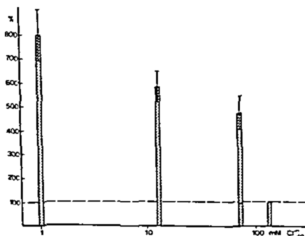


Fig 2 The relationship between $[Cl^-]_o$ and spontaneous mechanical activity The substituent in this experiment was NO_3^- Each bar is shown with ± 2 S E (n = 8) The values are expressed as percent of control activity

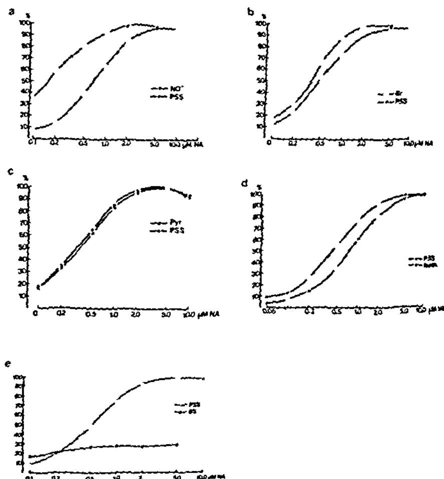


Fig 3 Dose response curves for noradrenaline in a) NO_3 b) Br c) pyruvate d) isethionate and e benzenesulphonate solutions Open circles Normal PSS filled circles test solutions $n = 8$

Results

The effect of anion substitution on spontaneous mechanical activity

Portal veins exposed to different anion substitutes exhibited a behaviour that was dependent on the type of anion and seemed to be related to their relative permeabilities Fig 1 shows the average spontaneous activity in each of the following anion solutions NO^3 Br pyruvate isethionate and benzenesulphonate expressed as percent of control values NO_3 increased the spontaneous mechanical activity 5 5 times while Br caused a 3 fold increase Pyruvate and isethionate both reduced the contractile activity to about 50 % of control while benzenesulphonate had a moderate effect The changed mechanical activity in all these solutions was usually the result of an increased frequency of contractions with an enhanced amplitude in NO_3 or Br solutions and a very much reduced contraction amplitude in the

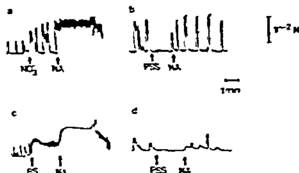


Fig. 4. a) The effect of noradrenaline 0.2 μM just after changing to NO_3 solution. b) The effect of noradrenaline in the same concentration in normal PSS after 15 min in NO_3 solution. c) and d) same as a) and b) in benzenesulphonate solution. BS = benzenesulphonate, NA = noradrenaline. Time and tension scales are indicated by the bars.

other solutions. Benzenesulphonate was an exception in that in this solution the contractions were normal in amplitude but with prolonged duration, while the contraction frequency was reduced to very low values. All effects of the substituted anions on spontaneous activity were fully reversible.

In another series of experiments the $[Cl]_o$ was reduced stepwise by substitution with NO_3 and the mechanical activity measured. The results are shown in Fig. 2. As can be seen total substitution of Cl by NO_3 increased spontaneous activity about 8 times, while a 50 % substitution caused a 5-fold increase. Thus the spontaneous activity is obviously related to the Cl_i/Cl_o gradient across the membrane. Substitution by Br gave similar results, but in this solution the increase in activity was less marked.

The effect of noradrenaline in anion substituted solutions

Dose response curves for noradrenaline were obtained by adding at 15 min intervals increasing doses of the drug to the muscle preparation. The response to the drug was measured as the difference between the mean tension during 3 min preceding drug administration and the mean tension during 3 min of drug application. Thus the spontaneous activity, which persists in a completely adrenergically

TABLE 1. The ratio between the initial effect of noradrenaline in anion substituted solution and the effect of the drug upon return to normal PSS

Substituent	NA = 0.5 μM (n = 8)	NA = 0.5 μM (n = 8)
Nitrate	2.27 ± 0.43	1.32 ± 0.41
Bromide	2.00 ± 0.35	1.4[illegible] ± 0.12
Pyruvate	2.41 ± 1.13	1.78 ± 0.39
Isethionate	5.29 ± 0.78	2.[illegible] ± 0.[illegible]
Benzenesulphonate	—	8.73 ± 2

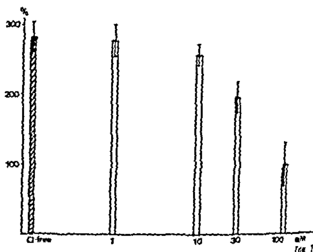


Fig 5 The relationship between [Cl⁻]ₒ and the effect of noradrenaline (0 2 μM) expressed as percent of control values Vertical bars indicate ± 2 S E (n = 8)

and cholinergically blocked muscle and is of non neural non humoral origin was subtracted from the total mean tension and only the extra tension induced by the drug entered the calculations Fig 3 a b c d and e shows the dose response curves for noradrenaline in different anionic solutions The muscles had been allowed equilibration in each solution for at least 30 min before noradrenaline was applied As shown in the figures the dose response curve in pyruvate solution was similar to that in normal PSS while in NO_3 and Br solutions the curves were shifted to the left and in isethionate solution to the right The increase in sensitivity in NO_3 solution was 4 3 times control and in Br solution 1 3 times control while in isethionate solution the drug sensitivity was reduced to 0 6 of control based on ED_{50} values The different anions thus influenced the noradrenaline induced contraction in the order $NO_3 >$ Br $>$ pyruvate $=$ Cl $>$ isethionate The dose response curve in benzene sulphonate solution differed greatly from the others in that the response to noradrenaline was small only 20 % of normal at all concentrations i e the response was not dose dependent

In another series of experiments the substituted anions were added only 2 min before noradrenaline stimulation and again noradrenaline was given 2 min after return to normal PSS when the muscles had been in an anion substituted solution for a total time of 15 min Fig 4 shows typical records obtained in NO_3 and benzenesulphonate solutions respectively The drug response was tested at 0 2 and 0 5 μM in these solutions The results are summarized in Table I expressed as the ratio between the response 2 min after introducing the foreign anion and the response 2 min after return to PSS All substituting anions increased the drug response after change to the substituted solution and decreased or abolished the response to the drug upon return to normal PSS Benzenesulphonate was the most effective and NO_3 the least In this respect the anions fell in the order $NO_3 \leq$ Br $\leq$ pyruvate $<$ isethionate $<$ benzenesulphonate

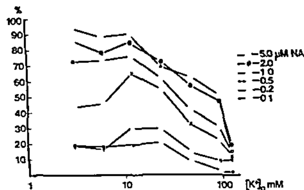


Fig 6 The effect of noradrenaline in solutions with increasing $[K]_0$ All values are expressed in per cent of the maximum response in normal PSS (n = 6)

A more detailed study was made on NO_3 and Br substitution In a series of experiments the $[Cl]_o$ was changed stepwise down to 1 mM by substitution with NO_3 or Br and the noradrenaline induced contraction tested in these conditions The results for NO_3 are summarized in Fig 5 which shows the mean tension induced by the drug in percent of control values versus log $[Cl]_o$ The effect of Cl reduction was maximal already at 10 mM and a further reduction to zero had no significant effect on the noradrenaline induced tension response The drug induced tension development in the substituted solutions was roughly inversely linearly related to log [Cl] down to 10 mM both for NO_3 and Br although the former ion gave a higher response at all concentrations

In all these experiments the basic idea was to increase the Cl gradient *i e* to augment the difference between E_m and E_{Cl} and thereby to test if—assuming a constant change in P_{Cl} induced by a certain drug concentration—the drug response was related to the abovementioned parameters It is also possible to decrease the difference between E_m and E_{Cl} this however cannot be done by further increasing [Cl] Instead [K] can be increased stepwise causing a progressive fall in E_m while neither external nor internal chloride is changed (Wahlstrom 1973 a) In a series of experiments this was tested by increasing [K] to 12 24 36 48 and 128 mM and applying varying concentrations of noradrenaline when the activity had reached a steady level in each solution The results are shown in Fig 6 The drug response decreased with increasing [K] and only about 15 % of the control response remained in 128 mM K The curves except for the lowest drug concentration show a linear decrease above 12 mM K in accordance with earlier findings (Axelsson *et al* 1967) that the membrane potential does not start to fall until [K] is increased above 10—20 mM It should be noted here that the sum of the K induced and the NA induced tension development was less in excess K than the response to a similar dose of noradrenaline in normal solution The decrease in the noradrenaline induced response is therefore not solely due to an increase in the tension level from which the drug response takes off

Discussion

The effect of replacing chloride by a foreign anion was studied in intestinal smooth muscle by Kuriyama (1963 1970) He found that all substitutes depolarize the membrane transiently, while the long lasting effect was a depolarization in SO_4 $C_6H_5SO_3$ and Br solutions and hyperpolarization in NO_3 and I solutions. These effects he explained by the relative permeabilities of the anions, NO_3 and I being more permeant than Cl and having equilibrium potentials more negative than the membrane potential affected it more strongly than Cl

Also after equilibration with the different anions it appears that the spontaneous activity in the rat portal vein is related to the permeability of the substituent. Thus spontaneous activity is highest in NO_3 which is the most permeant (Brading and Tomita 1968) and least in isethionate which is the least permeant This may be explained by assuming that NO_3 and Br substitute for Cl in membrane conductance while pyruvate isethionate and benzenesulphonate do so only to a very limited degree NO_3 and Br then give a lower membrane potential causing higher spontaneous activity The potentiating effect of NO_3 and Br was inversely related to the logarithm of the external Cl concentration The anions appear in the same order when it comes to their influence on the noradrenaline effect. If Cl (or more generally an anion) is the main ion responsible for the membrane depolarization caused by the drug as has been proposed (Wahlstrom 1973 b) then one would expect that with a more permeant anion the dose response curve for noradrenaline would be shifted to the left while the opposite would b the case for a less permeant anion The results obtained so far on the portal vein support this notion.

On the other hand when noradrenaline was tested just after changing to a foreign anion the order of potency was the opposite Benzenesulphonate had the greatest potentiating effect and NO_3 the smallest However in this condition $[Cl]_i$ is virtually unchanged and E_{Cl} is +20 mV NO_3 being very permeant and at the moment having an equilibrium potential very much in the negative would tend to offset the augmented depolarizing effect of an increase in Cl-efflux while the more impermeant anions have no such effect

In excess K the response to noradrenaline was progressively diminished although about 15 % still persisted in K high solution This part of the drug response probably involves release of internal Ca as an essential factor The rest of the response however was clearly membrane potential dependent and decreased with decreasing difference between E_m and E_{Cl}

The results presented in this paper are most easily explained by assuming that changes in spontaneous activity are due to variations in the membrane potential and that those variations reflect changes in anion distribution and permeability. Chloride is responsible for about 65 % of the membrane conductance (Wahlstrom 1973 a) and P_{Cl} and E_{Cl} therefore largely determines the response to noradrenaline Anions like NO_3 and Br may substitute for chloride in the membrane response to the drug the magnitude and direction of the response depending on their relative permeabilities and their distribution at the time of application The great sensitivity

of Cl on the membrane potential and in the noradrenaline induced excitation has been fully confirmed

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References

Axelsson J B Johansson O Jonsson and B Wahlstrom The effects of adrenergic drugs on electrical and mechanical activity in the rat portal vein *Bibl anat* (Basel) 1966 *8* 16—20

Axelsson J B Wahlstrom B Johansson and O Jonsson Influence of the ionic environment on spontaneous electrical and mechanical activity of the rat portal vein *Circulat Res* 1967 *21* 609—619

Brading A F and T Tomita Effect of anions on the volume of smooth muscle *Nature* (Lond) 1968 *218* 276—277

Kuriyama H The influence of sodium potassium and chloride on the membrane potential of the smooth muscle taenia coli *J Physiol* (Lond) 1963 *166* 15—28

Kuriyama H Effects of ions and drugs on the electrical activity of smooth muscle In *"Smooth Muscle"* Arnold Ltd London 1970 366—396

Wahlstrom B Ionic fluxes in the rat portal vein and the applicability of the Goldman equation in predicting the membrane potential from flux data *Acta physiol scand* 1973a 89 436—448

Wahlstrom B A study on the action of noradrenaline on ionic content and sodium, potassium and chloride effluxes in the rat portal vein *Acta physiol scand* 1973b *89* 522—530

Acta physiol scand 1974 92 412—415
From the Institute of Physiology University of Helsinki Finland

Transfer of TRH through the Placenta and Metabolism in the Fetus of the Guinea-Pig

By

HARRY LYBECK and PEKKA VIRKKUNEN

Received 9 May 1974

Abstract

LYBECK H and P VIRKKUNEN *Transfer of TRH through the placenta and metabolism in the fetus of the guinea pig* Acta physiol scand 1974 92 412—415

The permeability of the placenta to thyrotropin releasing hormone (TRH) and the fetal metabolism of this hormone were studied by giving iodine labelled synthetic TRH to pregnant guinea pigs during the first trimester The radioactivities of various tissue fluids (serum urine amniotic fluid and maternal bile) and tissue samples (kidney liver small intestine muscle and cortex) of both mother and fetus were counted The biological activity of the transferred synthetic TRH was measured in fetal serum by bioassay Direct measurement of placental permeability to TRH is presented In the mother the metabolism seems to be the same as in mice and rats (Redding and Schally 1971 Virkkunen Leppaluoto and Lybeck 1972 In the fetus the main elimination route of TRH is through the liver to bile in contrast to the rapid urinary excretion in the mother The half life of TRH in the fetal serum is about ten times as long as in the mother reflecting a different elimination mechanism Fetal tissue concentrations of TRH are comparable to those of the mother and so may trigger the endocrine development of the fetal thyroid adenohypophyseal system

The development and functional level of the fetal thyroid axis appears to be dependent on the maternal thyroid status (Maenpaa 1972) However direct information on placental permeability to thyrotropin relasing hormone (TRH) is poor Limited amounts of free T_3 and T_4 cross the barrier the rate being greater with T_3 (Ginsburg 1971) There is no evidence that TSH crosses the placenta in biologically significant amounts Kajihara *et al* (1972) reported that TRH administered i v to pregnant rats on the 20th day of gestation resulted in an increase in colloid droplets in the fetal thyroid

This paper deals with direct measurement of placental permeability to iodine labelled synthetic TRH in the guinea pig and of the fate of this hormone in the fetus

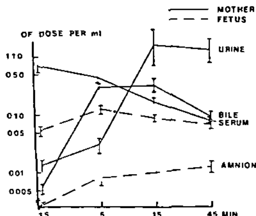


Fig 1 The amount of radioactivity in different body fluids after i.v administration of 125I TRH Mean±S E

Material and Methods

6 pregnant (20—25 days) guinea pigs (Orion Mankkaa) weighing 450—560 g were anesthetized with Nembutal® (Abbott England 30 mg/kg) and injected i v with 1 ng 125I TRH (12 mCi/μg) prepared by the chloramine T method (Virkkunen *et al* 1973) The fetuses (weighing 5 5—7 1 g) were sectioned 1 5 5 15 and 45 min later Serum and tissue samples of liver kidney small intestine (duodenum) muscle and occipital cortex were taken from the dam and fetuses In addition samples of maternal urine bile and fetal amniotic fluid were taken Radioactivities were measured with a β-counter (Berthold) Serum urine and bile samples were also submitted to low voltage paper electrophoresis for 4 h (veronal buffer pH 8 6 200 V) and the strips were counted with a 4π-scanner (Berthold) to detect any structural alternat on of the TRH molecule during elimination

Two pregnant (full term) guinea pigs weighing 1 2 and 1 4 kg were given 50 μg of synthetic TRH (Ferring Ltd Sweden) as before At 15 min blood samples were drawn into ice-cold tubes from the dam and fetuses After centrifugation at 4 C the separated serum was bioassayed immediately for TRH by a local modificat on of the McKenzie bioassay (Virkkunen 1974)

Results

The half life of 125I TRH in the maternal serum was 3 5 min (Fig 1) In the fetal serum radioactivity reached a maximum at 5 min thereafter having a half life of 40 min 125I TRH accumulated rapidly in maternal urine reaching a plateau between 15 and 45 min the concentration being about 10 times that in the serum Only small amounts of 125I TRH could be detected in the amniotic fluid and after 15 min the level had not reached even 1/100 of that in the maternal urine The labelled TRH soon appeared in maternal bile and the concentration was maximally 0 3 % of the dose/ml which is about one fifth of that in the urine

In connection with the relatively high radioactivity in the bile significant amounts of labelled TRH collected in the liver and duodenum of the dam (Fig 2) Greater amounts were seen only in the kidney (Fig 3) In the fetus the liver kidney and duodenum had closely similar elimination curves with maxima at 5 min and radioactivities comparable to that of the maternal duodenum

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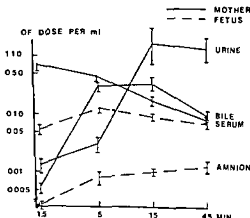


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Results

The half life of ¹ I TRH in the maternal serum was 3 5 min (Fig 1) In the fetal serum radioactivity reached a maximum at 5 min thereafter having a half life of 40 min ¹²⁵I TRH accumulated rapidly in maternal urine reaching a plateau between 15 and 45 min the concentration being about 10 times that in the serum Only small amounts of ¹ I TRH could be detected in the amniotic fluid and after 15 min the level had not reached even 1/100 of that in the maternal urine The labelled TRH soon appeared in maternal bile and the concentration was maximally 0 3 % of the dose/ml which is about one fifth of that in the urine

In connection with the relatively high radioactivity in the bile significant amounts of labelled TRH collected in the liver and duodenum of the dam (Fig 2) Greater amounts were seen only in the kidney (Fig 3) In the fetus the liver kidney and duodenum had closely similar elimination curves with maxima at 5 min and radioactivities comparable to that of the maternal duodenum

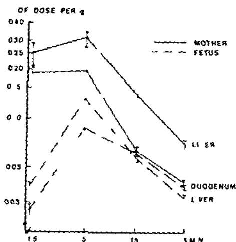


Fig 2

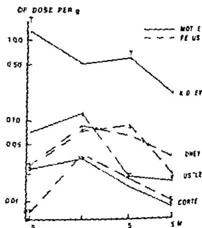


Fig 3

Fig 2 The radioactivity of maternal and fetal tissues after i v administration of ¹²⁵I TRH Mean ± S E

Fig 3 The radioactivity of maternal and fetal tissues after i v administration of ¹²⁵I TRH Mean ± S E

The muscle and brain cortex accumulated almost identical amounts of the dose in the fetus and dam (Fig 3) After 15 min the fetus had even higher concentrations of ¹ I TRH in both tissues

When non radioactive TRH was similarly given the values in the serum at 15 min were 0 06 % of the dose/ml in the mother and 0 012 % in the fetus

Discussion

No direct studies on placental permeability to TRH have previously been published In the rat however D Angelo Wall and Bowers (1971) have shown that the fetal adenohypophysis is able to release TSH in response to large amounts (250—500 μg) of TRH given s c to the mother during the perinatal period Kajihara et al (1972) have found raised colloid droplet formation in the rat fetal thyroid after administration of synthetic TRH i v to the mother at a dose level of 10 μg

Both labelled radioactive and synthetic TRH were used in this direct study of the placental permeability to TRH

The metabolism of i v given radioactive ¹ I TRH seems to be the same as in mice (Virkkunen Leppaluoto and Lybeck 1972) In both species the half life in maternal serum was 3 5 min In the guinea pig fetus however this was 40 min reflecting a slower elimination mechanism than in the mother In addition to a poorer renal function differences may exist in the rate of serum inactivation which plays an important role in regulating the biologically active TRH in the organism (Bassiri and Utiger 1972) The trace amount of ¹ I TRH in the amniotic fluid is evidence of the undeveloped renal function in the fetus

Instead of secretion of ^{1} I TRH into fetal urine the hormone is eliminated prin cipally through the liver into the bile thus increasing the radioactivity in the duodenum In the mother the main route of elimination of TRH is by secretion into the urine as in mice (Virkkunen Leppaluoto and Lybeck 1972) and man (Leppa luoto Virkkunen and Lybeck 1972)

The results of studies with pregnant guinea pigs dosed with native TRH support the finding that TRH crosses the placenta readily in biologically active form

The relatively high radioactivity in the fetal cortex soon after i v administration of ^{1} ^{1}I TRH makes it possible to trace the effect of maternal TRH on endocrine development and function in the fetus

References

BASSIRI R and R UTIGER Serum inactivation of immunological and biological activity of thyrotrop n releasing hormone (TRH) *Enodcrinology* 1972 *91* 657—664

D ANGELO S A N R WALL and C Y BOWERS Maternal — fetal endocrine Interrelations Demonstration of TSH release from the fetal hypophysis in pregnant rats administrated synthetic TRH *Proc Soc exp Biol* (N Y) 1971 *137* 175—178

GINSBURG J Placental drug transfer *Ann Rev Pharmacol* 1971 *11* 387—407

KAJIHARA A A KOJIMA T ONAYA Y TAKEMURA and T YAMADA Placental transport of thyrotropin releasing factor in the rat *Endocrinology* 1972 *90* 592—594

LEPPALUOTO J P VIRKKUNEN and H LYBECK Elimination of TRH in man *J clin Endo crin* 1972 *35* 477—478

MAENPAA J Congenital hypothyroidism Aetiological and clinical aspects *Arch Dis Childh* 1972 *47* 914—918

REDDING T W and A V SCHALLY The distribution of radioactivity following the administra tion of labelled thyrotropin relasing hormone (TRH) *in rats and mice Endocrinology* 1971 *89* 1075—1081

VIRKKUNEN P Some factors influencing TRH bioassay in mice *Acta endocr* 1974 *75* 452—460

VIRKKUNEN P J LEPPALUOTO and H LYBECK The distribution and elimination of 1—I labelled thyrotrop n releasing hormone in the mouse *Horm Metab Res* 1972 *4* 506—507

VIRKKUNEN P J LEPPALUOTO T RANTA and H LYBECK Preparation of ^{12} I labelled th rotropin releasing hormone *Horm Metab Res* 1973 *5* 58

Acta physiol scand 1974 92 416—420

From the Institute of Physiology University of Helsinki Finland

A Serum Factor Influencing the Physiological Activity of Thyrotropin-Releasing Hormone (TRH)
I Studies with human serum

By

PEKKA VIRKKUNEN

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Abstract

VIRKKUNEN P *A serum factor influencing the physiological activity of thyrotrop[in] releasing hormone (TRH) I Studies with human serum* Acta physiol scan[d] 1974 92 416—420

Thyrotropin releasing hormone was incubated at 2 C 37 C and 60 C with varying di[lu]tions of human serum and with serum fractions obtained by gel filtration The amount of TR[H] left after incubation was measured by bioassay The inactivation of TRH was shown to [be] complete by 90 min the half time being 18 min Dilution of serum more than 1 16 did [not] abolish TRH Binding of TRH to serum proteins could not be detected Instead of th[is] evidence is submitted for the enzymic nature of the serum protein which inactivates TR[H] In gel filtration this protein migrates between the globulins and albumin which sugges[ts a] molecular weight of about 100 000

The inactivation of a hormone is an important factor in the regulation of it[s] physiological activity in the organism That serum *in vitro* abolishes the biologica[l] activity of TRH of hypothalamic origin was demonstrated before the structure of this hormone had been worked out (Redding and Schally 1968) Later when the structure of TRH was established (Burgus *et al* 1969 Boler *et al* 1969) serum wa[s] found to inactivate the hormone by a deamination mechanism (Nair Redding and Schally 1971) The reactivation of serum inactivated TRH at a high incubation temperature could mean however that TRH formed complexes with a serum protein from which it was released during heating (Bassiri and Utiger 1972)

Although most of the evidence to date supports the view that TRH is inactivated by deamination it seemed necessary to study the interaction of serum and TRH b[y] another technique and to compare the inactivation process *in vitro* with the behaviour of TRH *in vivo* (Leppäluoto Virkkunen and Lybeck 1972)

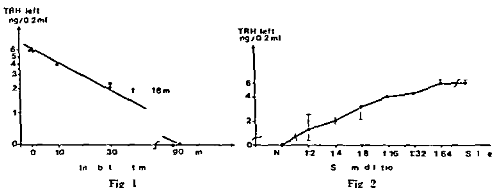


Fig 1 The amount of TRH left after incubation with serum as a funct on of incubation t me

Fig 2 The amount of TRH left after incubation with various serum dilutions Mean ±S.D

Material and Methods

Incubation of TRH and serum TRH (Ferring Ltd) was added (36 ng in 20 μl saline) to the following freshly prepared solutions human sera, 0 9 NaCl (saline) serum diluted with saline and serum fractions so that the TRH concentration in each was 30 ng/ml (see below) Serum samples were collected from the same subjects for each experiment. The plastic test tubes were agitated in a water bath at 37 C for 0 10 30 and 90 min At 2 C and 60 C the incubation time was only 90 min In a single experiment serum containing TRH was incubated wiht para-chloromercuribenzoate (10 ^{4} M) at 37 C for 90 min At the end of the incubation period 0 2 ml al quots were immediately injected into bioassay mice prepared 24 h before (see below)

Gel filtration of serum 20 ml of serum was submitted to gel filtrat on immediately after col lection (Sephadex G 200 bed volume 200 ml elution rate 1 ml/m n) at room temperature (20—22 C) Fractions of 4 ml were collected and the r opt cal densities measured at 280 n m. The proteins were divided into four equal portions with relative (to void volume) elution volumes of 1 0—1 3 1 3—1 6 1 6—1 9 and 1 9—2 2 (fraction 1 0—1 3 consisted mainly of globulins and fraction 1 9—2 2 of albumins as judged from electrophoresis) For incubation stud es d luted fractions were concentrated fi e fold with a Millipore filter system (molecular cut off 1000 daltons)

The complexing of TRH to serum proteins was evaluated by adding unlabelled TRH or ^{125}I TRH (Virkkunen *et al* 1973) to the serum and immediately after mixing submitting the sample to gel filtration The biological acti ity and radioactivity of the 4 fractions and of the rest of the eluate (up to a relati e eluation vloume of 2 2—4 0) were measured

Bioassay of TRH Female mice weighing 15—20 g were fed on a low iodine diet and d stilled water for 2 weeks and kept at 7 C for the last 5 days 24 h before the assay the m ce were injected with 0 1 μg of T_3 and 3 μCi of ^{131}I s c At the beginning of the assay a 0 1 ml blood sample was withdrawn retro-orbitally and a 0 2 ml ample was injected i v Two h later the second blood sample was withdrawn The ad oactivities of the first (x) and second (y) samples were measured and the response was e pressed as log y—log x + 2 In each assay TRH standards of 2 6 18 and 54 ng were used and the TRH content of the unknown sample was estimated graphically

Results

Incubation of synthetic TRH with human serum for various periods and at different temperatur s TRH was incubated at 37 C with human serum or saline (30 ng/ml) for 0 10 30 and 90 min At the end of the incubation period the TRH content of

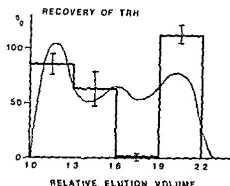


Fig 3

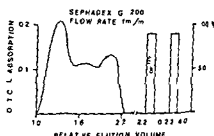


Fig 4

Fig 3 Inactivation of TRH by various fractions of human sera Mean ±S D

Fig 4 Gel filtration of serum and TRH or ¹ I TRH mixtures

the mixtures was bioassayed Incubation of TRH with serum significantly lowered the TRH content in as little as 10 min and the response was completely abolished by 90 min Incubation with saline did not alter the response In three different experiments the half time of TRH in human serum *in vitro* was 18 min (Fig 1) No significant inactivation of TRH by serum could be seen in mixtures incubated at *2° C or 60° C for 90 min (Table I)*

Incubation of TRH with various serum dilutions TRH was incubated as before for 90 min with serum and saline and their dilutions from 1 2 to 1 64 in 4 different expts The TRH content of the dilution was then bioassayed The remaining TRH (in ng) was lowest after incubation with 1 2 serum dilution and rose gradually with increasing proportions of saline Serum diluted more than 1 16 did not abolish the biological activity of TRH (Fig 2)

Incubation of TRH with various serum protein fractions The four protein fractions obtained by gel fractionation were incubated with TRH for 90 min as before and the remaining TRH content was bioassayed in 4 different expts The first and last fractions (1 0—1 3 and 1 9—2 2) containing globulins and albumins did

TABLE I Effect of various treatments on the inactivation of TRH by human serum *in vitro*

Treatment	*Response in bioassay* (mean ± S D)
Incubation of TRH with saline at 37 C	2 ?f ± 0 28
serum	2 4? ± 0 14
2 C	2 8 ± 0 1?
60 C	2 80 ± 0 17
and pCMP	2 ?9 ± 0 1?

pCMB = para chloromercuribenzoate 10 M

not inactivate TRH whereas the mid fractions clearly did so fraction 1 6—1 9 being the most potent and completely inactivating the added TRH (Fig 3)

Incubation of TRH and serum with p chloromercuribenzoate At 10^{-4} M concentration the enzyme inhibitor p-chloromercuribenzoate significantly inhibited inactivation of TRH by serum (Table I)

Gel filtration of the mixture containing serum and TRH TRH synthetic or radioactive was added to serum and the mixture was submitted to gel filtration immediately as before The protein fractions gave no response in TRH bioassay and their radioactivity was slight whereas the rest of the eluate (relative elution volume 2 2—4 0) showed a TRH response of 80 % when unlabelled TRH was added and of 91 % when ^{125}I TRH was added (Fig 4)

Discussion

In the present study the in vitro inactivation of TRH by incubation with human serum at 37° C was complete by 90 min and the half life of TRH in serum was 18 min Previously Redding and Schally (1969) using TRH bioassay and rat plasma and Bassiri and Utiger (1972) using TRH immunoassay and human serum obtained similar results In conformity with Blackwell and Guillemin (1973) we could not show significant inactivation of TRH by serum after incubation at 2° C or 60° C

The observations of Nair Redding and Schally (1971) and Redding and Schally (1971) suggest that the inactivation process is enzymic and occurs by deamination of the proline amide We have previously demonstrated that in vivo the disappearance of TRH from human plasma occurs in 2 stages an early rapid one (half life 5 min during 1—5 min) and a relative slower one with a common half life of 12 min (during 1—15 min) (Leppaluoto Virkkunen and Lybeck 1972) Assuming the inactivation is as rapid in vitro as in vivo the difference between the half lives (12 vs 18 min) is evidently due to an additional inactivation mechanism in vivo An explanation was offered by Bassiri and Utiger (1972) who suggested that TRH may be bound to some serum protein We were able to show however that virtually all the TRH added to serum was recovered separately from the serum proteins in gel filtration and that the biological activity of TRH was completely abolished by particular serum fraction We could also prevent the inactivation of TRH by incubating the serum with SH-enzyme inhibitor or at low or high (2° C and 60° C) temperature These findings strongly suggest that serum inactivation of TRH is enzymic and that TRH is not bound to serum proteins

In their original paper Redding and Schally (1968) found that TRH was inactivated by albumin and globulin fraction separated by precipitation techniques In the present study these fractions separated by gel filtration did not have this property whereas a certain fraction between them did inactive TRH So we do not think that albumins and globulins are associated with the process of TRH inactivation

TRH was shown to be excreted in large amounts in the urine of normal subjects (Leppaluoto Virkkunen and Lybeck 1972) a finding which would probably explain the discrepancy in the half lives From those data we calculated that by 50 min half the injected TRH had been excreted into the urine By combining (adding the inverse values) the half life of TRH in vitro (18 min) with that seen in kidney excretion (50 min) we obtain a theoretical half life of TRH in circulating plasma 12 min Our finding that the excretion TRH by the kidney is linked with the effective half life of TRH in vitro is supported by a recent paper (Barcena *et al* 1973) These authors found increased TSH responses to TRH in patients with impaired kidney function

According to the present investigation the serum protein possibly an enzyme that inactivates TRH migrates in gel filtration between the globulin and albumin fractions which suggests a molecular weight of about 100 000 Although inactivation seems to be as effective in vitro as in vivo simultaneous measurements for instance of biological activity and radioactivity of the TRH administred is recommended

References

BARCENA D A KASTIN S SCHALCH M ZAMORA E PASTEN A KATO and A SCH Responses to thyrotropin releasing hormone in patients with renal failure and after in normal men *J clin Endocr* 1973 *36* 117—120

BASSIRI R and R UTIGER Serum inactivation of the immunological and biological ac of thyrotropin releasing hormone (TRH) *Endocrinology* 1972 *91* 657—664

BLACKWELL R E and R GUILLEMIN Hypothalamic control of adenohypophysial secr *Ann Rev Physiol* 1973 *35* 357—390

BOLER J F ENZMANN K FOLKERS C Y BOWERS and A V SCHALLY The identi chemical and hormonal properties of the thyrotropin releasing hormone and pyrogluta histidyl proline amide *Biochem biophys Res Commun* 1969 *37* 705—710

BURGUS R T DUNN D DESIDERIO and R GUILLEMIN Derives polypeptidiques de synt doues d activite hypophysiotrope TRF *C R Acad Sci* (Paris) 1969 *69* 226—228

LEPPALUOTO J P VIRKKUNEN and H LYBECK Elimination of TRH in man *J cl* *E crin* 1972 *35* 477—478

NAIR R M G T W REDDING and A V SCHALLY Site of inactivation of thyrotr releasing hormone by human plasma *Biochemistry* 1971 *10* 3621—3624

REDDING T W and A V SCHALLY Studies on the inactivation of thyrotropin releasing fa (TRF) *Fed Proc* 1968 *27* 823

REDDING T W and A V SCHALLY Studies on the thyrotropin releasing (TRH) activ peripheral blood *Proc Soc exp Biol* (N Y) 1969 *131* 415—425

REDDING T W and A V SCHALLY The distribution of radioactivity following the admin tion of labelled TRH in rats and mice *Endocrinology* 1971 *89* 1075—1079

VIRKKUNEN P J LEPPALUOTO T RANTA and H LYBECK Preparation of I label thyrotropin releasing hormone *Horm Metab Res* 1973 *5* 58

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From the Department of Anatomy University of Helsinki Finland

The Acid Catalyzed Formaldehyde Induced Fluorescence in the Hypophysis of the Human Fetus

By

SEPPO PARTANEN[1]

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Abstract

PARTANEN S *The acid catalyzed formaldehyde induced fluorescence in the hypophysis of the human fetus* Acta physiol scand 1974 92 421—426

The acid catalyzed formaldehyde induced fluorescence (FIF) was studied in Epon embedded hypophyses of human fetuses aged between 12 and 19 weeks To test the validity of Epon embedded sections in glacial acetic acid induction of FIF sections of the hypophyses of adult rats were used It was observed that Epon embedded sections were suitable After acidification a large number of fluorescent cells was observed in the pars distalis of the hypophyses of human fetuses new fluorescent cells appeared and the fluorescence intensity in the cells already fluorescent after the ordinary FIF increased On the basis of this increase the fluorogenic substance is probably the same as in other mammals i e a peptide with NH terminal tryptophan This peptide has been proposed to be somehow involved in the storage and/or release of protein hormones The fluorescence is probably a sign of maturity of secretory granules in large number of PAS positive cells in the pars distalis of the fetal hypophysis First fluorescence after acidification was observed in the cells of the pars intermedia at the age of 15 weeks At the age of 19 weeks most cells exhibited yellow fluorescence The fluorescence intensity was weaker than in the cells of the pars distalis Thus the pars intermedia of man does not differ in this respect from that of other mammals

The cells of the pars intermedia and some cells of the pars distalis of the hypophyses of various mammals exhibit specific formaldehyde induced fluorescence (FIF) indicating the presence of a fluorogenic substance (Pearse and MacGregor 1964 Dahlström and Fuxe 1966 Björklund and Falck 1969 a b) Fluorescent cells are also present in the pars distalis of the hypophysis of the human fetus but not in the pars intermedia during the first 19 fetal weeks (Partanen and Hervonen 1973) The fluorogenic substance is stored in PAS positive cells known to produce FSH LH and TSH in the pars distalis and MSH in the pars intermedia (Dahlström and Fuxe 1966) PAS positive cells of the pars intermedia produce also ACTH (see Kraicer Gosbee and Bencosme 1973) The concepts of the nature of the fluorogenic substance have changed during recent years the present concept favors on the basis of histochemical microspectrofluorometric and chemical studies a peptide

[1] This work was supported by a Grant for Young Research Workers University of Helsinki

with NH terminal tryptophan (Hakanson *et al* 1972) The fluorescence intensity of this tryptophyl peptide or peptides and that of the cells of the adenohypophysis increases if the formaldehyde treatment is modified 1) by oxidation with ozone gas or 2) by acidification with hydrochloric acid or with glacial acetic acid (Bjorklund and Falck 1969 b Hakanson and Sundler 1971 Hakanson *et al* 1972)

The aim of this study was to demonstrate acid catalyzed FIF in the adenohypophysis of the human fetus Special attention was directed to the cells of the pars intermedia which exhibited no specific fluorescence after the ordinary FIF

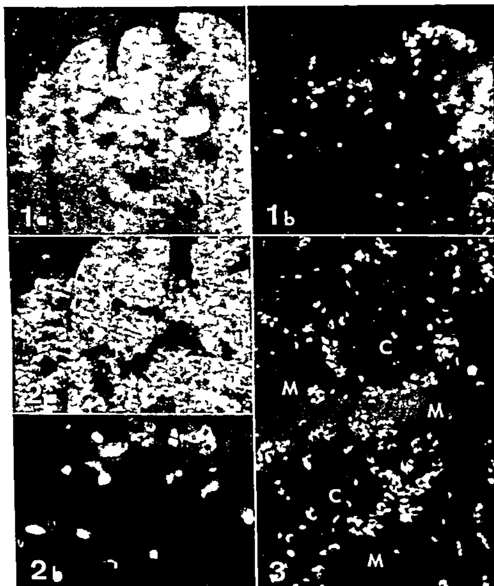
Material and Methods

7 human fetuses were obtained from legal interruptions of pregnancy performed by laparotomy The CR lengths (centimeters) and estimated ages (weeks) were 6 0 (11 8) two 7 5 (13) 9 8 (15 0) 9 9 (15 0) 10 5 (15 5) and 14 3 (18 7) The hypophyses were prepared within 1—3 min after the disconnection of the fetomaternal circulation Then the hypophyses were processed for the histochemical demonstration of catecholamines by *FIF method* (Eranko 1967) The specimens were embedded in Epon resin except one CR length 6 0 cm which was embedded in paraffin From Epon embedded hypophyses sections were cut at 5 *µm* with a Pyramitome from paraffin embedded at 7 *µm* with a microtome Sections were mounted on microscope slides with xylene and covered with a cover glas For fluorescence studies a Leitz Orthomat microscope fitted with an appropriate filter combination for catecholamine studies was used The colour of catecholamine fluorophore appeared blue green that of serotonin yellow After examination and photography xylene was allowed to evaporate from Epon embedded sections The paraffin embedded sections were deparaffinized after removing the cover glass with a small amount of xylene and plotting paper then the cover glass was reinserted Thereafter the sections were mounted in glacial acetic acid for 5—30 min (Bjorklund and Falck 1969 b) and sections were reexamined and rephotographied For general orientation some sections were stained with 1 % Toluidine Blue Sections of Epon or paraffin embedded hypophyses of adult rats were prepared under identical methodological conditions to test the validity of the acidification procedure in Epon embedded specimens

Results

The increase of the fluorescence intensity in the cells of the pars intermedia of the adult rat hypophyses was clear during mounting of sections in glacial acetic acid. This was observed both in paraffin and Epon embedded sections The mounting time of 15—30 min was sufficient to produce a maximal increase of the fluorescence intensity The appearance of new fluorescent cells in the pars distalis was demonstrated both in paraffin and Epon embedded sections Thus glacial acetic acid can penetrate in Epon resin to react with the reaction product of fluorogenic substance formed in the FIF reaction and produces an increase in the fluorescence intensity The fluorescence faded slower in the UV light after acidification than after the ordinary FIF reaction The mounting time of 15—30 min was chosen for the sections of the human hypophyses

Pars distalis of the human hypophysis In all ages studied the fluorescence intensity increased during the acidification procedure in the cells fluorescent already after the ordinary FIF reaction Also the acidification induced the appearance of a moderate number of new fluorescent cells in all ages (Figs 1 2) The colour of the fluorescence was yellow and appeared granular at higher magnification The fluor



Figs 1 and 2 1 (a) The pars distalis of the hypophysis of 1? week-old human fetus Paraffin embedded section Some ells exhibit weak yellow fluorescence (in the right uppe corner) after the ord nary FIF method 1 (b) The same section as in Fig 1 (a) after mounting in glacial acetic acid The fluorescence inten ity and the number of the fluorescent cells are i creased X 81 ? a) and b H gher magnification of same field as in Fig 1 (a) and 1 (b) respecti l Afte mounting in glacial acet c acid some cells exhibit strong yellow fluorescenc strictl localized to the cytoplasm X 185 A fixation point in Fig 1 and 2 is indicated by an ast r sk

Fig 3 Th pars d stal of the h pophysis of 15 5 week-old human fetus Epon embedded sect on Aft r m unt ng in glacial t c acid large number of yellow fluorescent cells is observed mainl n tl pe iph ry of the cells cords (C) The mesenchymal tissue (M) X 81

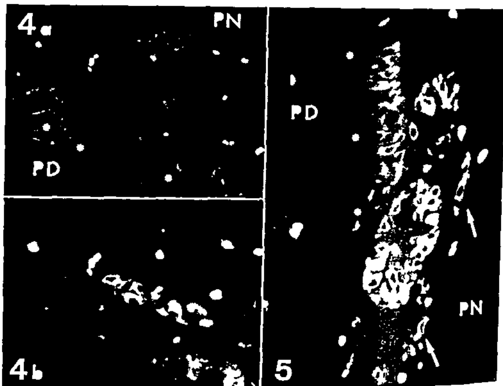


Fig 4 4 (a) The pars intermedia of the hypophysis of 15 5 week-old human fetus An Epon embedded section after the ordinary FIF method The cells of the pars intermedia show no specific fluorescence 4 (b) The same section after mounting in glacial acetic acid Some cells of the pars intermedia exhibit medium strong yellow fluorescence The pars distalis (PD) the pars nervosa (PN) The hypophyseal cleft is indicated by asterisks X 185

Fig 5 The pars intermedia of the hypophysis of 19 week-old human fetus Epon embedded section The cells of the pars intermedia exhibit after mounting in glacial acetic acid ellow fluorescence the intensity varies from weak to moderate strong Arrows indicate fluorescent cells penetrating into the pars nervosa Abbreviations as in Fig 4 (a) X 185

escence was strictly restricted in the cytoplasm It seemed that after the acidification the number of new fluorescent cells was larger at younger age groups Thus the number of the fluorescent cells after the acidification seemed to be fairly equal per unit area of the glandular cells during the period studied However it was difficult to ascertain the number of the fluorescent cells in the human hypophyses because the amount of the mesenchymal tissue varied in various parts of the hypophy es and in different ages

After the acidification the fluorescent cells were dispersed evenly over the whole pars distalis and their number in the periphery of the cell cords was larger than in the centre (Fig 3) The number of the fluorescent cells was large

Pars intermedia of the human hypophysis The pars intermedia consisted in all specimens studied of some layers of tall cells It was separated from the pars di tal s by the hypophyseal cleft and from the pars nervosa by mesenchymal tissue Between

the plate of the cells of the pars intermedia and the pars nervosa some cell of the pars intermedia were regularly seen to form tubular cell cords First weakly fluorescent cells after the acidification were seen at the age of 15 weeks The fluorescence was hardly visible and was located in the cells facing the pars nervosa and in tubular cell cords (Fig 4) In the older fetuses the number and intensity of the fluorescent cells increased and at the age of 19 weeks most cells exhibited a moderately strong yellow fluorescence (Fig 5) However the fluorescence intensity was weaker than that in the fluorescent cells of the pars distalis

Discussion

The present histochemical findings suggest that the fluorogenic substance in the cells of the human adenohypophysis is the same as in other mammals *i e* a peptide with NH_2-terminal tryptophan

The function of this tryptophyl peptide in the storage and secretion of protein hormones is unknown In the C cells of the thyroid gland of cat tryptophyl peptide disappeared simultaneously with the depletion of granules and calcitonin by the vitamin D It was suggested that tryptophyl peptide is somehow involved in the storage and secretion of the protein hormone (Hakanson *et al* 1973) The depletion of intracellular monoamine (serotonin) from the parafollicular cell of the thyroid gland of bat by reserpine caused morphological changes in the mature secretory granules (Gershon and Nunez 1973) This might also represent some connection between the intracellular monoamines and the storage of the protein hormones in the granules The ame possible connection between tryptophyl peptide and secretory granules of PAS positive cells of the adenohypophysis seems obvious

The increase in the number of the fluorescent cells in the pars distalis during the period studied as was seen after the ordinary FIF (Partanen and Hervonen 1973) was not so clear in the acid catalyzed FIF Thus the accumulation of tryptophyl peptide seems to occur before the age of 13 weeks The large number of the fluorescent cells during the period studied migh indicate the presence of mature secretory granules in large number of PAS-positive cells *i e* FSH LH and TSH producing cells

The accumulation of tryptophyl peptide in the cells of the pars intermedia occurred after the age of 15 weeks *i e* later than in the pars distalis The pars intermedia is weakly developed in man and in the adults it is composed of cysts filled with colloid and lined with PAS positive cells Some of these cells penetrate into the pars nervosa (see ref Diepen 1962) However it was possible to detect fluorescence in the cells of the pars intermedia of the hypophyses of human fetuses Thus the cells do not differ from the cells of other mammals in this respect The late accumulation of fluorogenic substance may reflect in one way poor development of the pars intermedia in man In the hypophyses of rodents which possess well developed pars intermedias the accumulation of fluorgenic substance in the cells occurred earlier and in larger am unt than in the pars distalis (Partanen and Rechardt 1973)

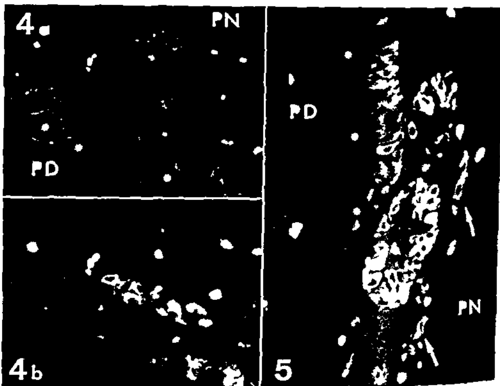


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From the Institute for Nutrition Research University of Oslo the Institute for Medical Biology University of Tromso Norway and the College of Biological Science University of Guelph Ontario Canada

How do Seal Pups Survive Birth in Arctic Winter?

By

H J GRAV A S BLIX and A PASCHE

Each year in February and March thousands of harp seal pups (*Pagophilus groenlandicus* (Erxleben 1777)) are born on the open icefloes of the North Atlantic Ocean The weather conditions are often severe with recorded air temperatures as low as $-30°$ C This implies that the pup is exposed to a thermogradient approaching 70° C at the very moment of birth The thermoshock incurred may further be potentiated by the arctic wind often playing in concert with the low temperature In the harp seal the thick layer of subcutaneous blubber is a postnatal development and is aquired in the course of the suckling period at birth no such layers is evident (Sivertsen 1941) This implies that the wet newborn pup in addition to the unfavourable climatic conditions also has to contend with a deficiency in adequate insulation Survival would thus appear unlikely in the absence of a suitable adaptive change

In the search for the nature of this adaptation we decided to look for the occurrence of brown adipose tissue in newborn pups of the harp seal Non shivering thermal activity arising from the brown adipose tissue is known to provide the thermogenic basis for survival in the immediate postnatal period in several species of small terrestial mammals (Smith and Horwitz 1969) Thus about 70 % of the total thermogenic defence against cold (as required for maintenance of homeothermia) has been shown to arise from the brown adipose tissue of newborn rabbits (Dawkins and Hull 1964)

We have recently made careful studies of 3 newborn harp seal pups weighing 10—14 kg from the Newfoundland stock None of the animal examined had developed subcutaneous white blubber Immediately below the skin the carcass was convoluted in adipose tissue of a type differing in appearance from blubber It was yellowish brown in colour and formed a continuous layer which varied in thickness from 8 mm in the nucal and sacral regions to 2 mm in the occipital region Small deposits of similar tissue were found intramuscularly around the neck and in the axillae and as thin sheets surrounding the veins of the pericardium Characteristically the flippers which serve a function in counter-current heat exchange with the environment lacked adipose tissue

References

BJORKLUND A and B FALCK Pituitary monoamines of the cat with special reference of an unidentified monoamine like substance in the adenohypophysis *Z Zellforsch* 1969 a *93* 254—264

BJORKLUND A and B FALCK Histochemical characterization of a tryptamine like substance stored in cells of the mammalian adenohypophysis *Acta physiol scand* 1969 b *77* 475—489

DAHLSTROM A and K FUXE Monoamines and the pituitary gland *Acta endocr* (Kbh) 1966 *51* 301—314

DIEPEN R Der Hypothalamus In *Handbuch der mikroskopischen Anatomie des Menschen* *VII* (Ed W Bargmann) pp 183—206 Berlin Gottingen Heidelberg Springer 1962

ERANKO O The practical histochemical demonstration of catecholamines by formaldehyde induced fluorescence *J roy micr Soc* 1967 *87* 259—276

GERSHON M D and E A NUNEZ Subcellular storage organelles for 5 hydroxytryptamine in parafollicular cells of the thyroid gland *J Cell Biol* 1973 *56* 676—689

HÅKANSON R and F SUNDLER Formaldehyde condensation A method for the fluorescence microscopic demonstration of peptides with NH terminal tryptophan residues *J Histochem Cytochem* 1971 *19* 477—482

HÅKANSON R L I LARSSON A NOBIN and F SUNDLER Tryptamine or tryptophyl peptides in endocrine cells of the mammalian adenohypophysis? *J Histochem Cytochem* 1972 *20* 908—916

HÅKANSON R A MELANDER CH OWMAN and F SUNDLER Depletion of secretory granules calcitonin and formaldehyde ozone induced fluorescence from cat thyroid C cells by vitamin D treatment *Histochemie* 1973 *36* 89—96

KRAICER J J L GOSBEE and S A BENCOSME Pars intermedia and pars distalis Two sites of ACTH production in the rat hypophysis *Neuroendocrinology* 1973 *11* 156—176

PARTANEN S and A HERVONEN Monoamine containing structures in the hypothalamo-hypophyseal system in the human fetus *Z Anat Entwickl Gesch* 1973 *140* 53—60

PARTANEN S and L RECHARDT Histochemically demonstrable monoamines in the pituitary gland and median eminence of the female rat during the postnatal development *Z Zellforsch* 1973 *147* 41—57

PEARSE A G E and M M MACGREGOR Functional cytology of the pituitary gland *Ann Rep Brit Cancer Camp* 1964 *2* 655—606

development of a cold skin required for an aquatic life in a cold sea reaches its completion at the final moult when the seal is aged about 30 days Prior to this final developmental change Irving (1973) considers that defence against a cold environment resides in the fur and in the steadily increasing blubber The infantile fur of the harp seal insulates efficiently when dry but poorly when wet (Irving 1973) the subcutaneous blubber is all but absent at birth Furthermore very young pups have not been observed to shiver even when wet (unpublished observation) Thus at the time of delivery the pup possesses none of the accepted defence mechanisms against heat loss neither can it raise its metabolism by way of shivering Compensation for heat losses therefore has to take the form of elevated rates of metabolic or non shivering heat production

In agreement with this Davydov and Makarova (1964) have shown that the increase in oxygen consumption rates in response to immersion in cold water was very high for harp heal pups aged 0—7 days The magnitude of metabolic increment upon submersion diminished steadily as the pups grew older and successively developed a cooler skin After the moult to the wettable adult skin the pup had completely lost its non shivering thermal response in ice water

It is tempting to propose that the pups meet the thermal challenge offered at birth by stimulating oxidation of the lipid stores contained in the brown adipose tissue the terminal stimulus for this effect being the drop in external temperature Having survived the initial thermal shock the pups replenish their lipid stores by suckling The unique composition of the harp seal milk (Sivertsen 1941) containing about 40 % (W/W) of triglycerides makes it most suitable as source both for the ensuing rapid accumulation of subcutaneous blubber and for maintenance of non shivering thermogenesis As the blubber insulation thickens in the course of the following weeks a high rate of heat production in brown adipose tissue would successively become superfluous

References

ANDERSEN H T E N CHRISTIANSEN H J GRAV and J I PEDERSEN Thermogenesis of brown adipose tissue reflected in mitochondrial respiratory control *Acta physiol scand* 1970 *80* 1—10

DAVYDOV A F and A R MAKAROVA Changes in heat regulation and circulation in new born seals on transition to aquatic form of life *Fed Proc* 1964 *24* II T563—T566

DAWKINS M J R and D HULL Brown adipose tissue and the response of new born rabbits to cold *J Physiol* (Lond) 1964 *172* 216—238

HULL D The structure and function of brown adipose tissue *Brit med J* 1966 22 92—96

IRVING L Aquatic mammals Pp 47—96 in *Comparative Physiology of Thermoregulation* Vol III Ed G C Whittow New York Academic Press 1973

SIVERTSEN E Biology of the harp seal *Hvalrådets Skrifter Norske Vidnskaps Akad Oslo* 1941 No 26

SKAANE O E N CHRISTIANSEN J I PEDERSEN and H J GRAV Oxidative properties of brown adipose tissue mitochondria from rats guinea pigs and hedgehogs *Comp Biochem Physiol* 1972 *42B* 91—107

SMITH R E and B A HORWITZ Brown fat and thermogenesis *Physiol Rev* 1969 *49* 330—425

Acta physiol scand 1974 92 430—432
From the Institute of Medical Chemistry Biomedical Center Uppsala Sweden

(^{32}P)Phosphoryl Transfer by Endogenous Protein Kinase at the Glia and Glioma Cell Surface in Culture into Extrinsic Acceptor Proteins

By

GUNNAR ÅGREN and GUNNAR RONQUIST

Protein kinase (ATP protein phosphotransferase EC 2 7 1 37) has been established to be associated with the plasma membrane of different cells (Ågren and Ronquist 1970 1971 1974 Ronquist and Ågren 1970 Schlatz and Marinetti 1971 Kinzel and Mueller 1973 Lemay *et al* 1974)

At least part of this plasma membrane activity is located at the external surface of the membrane (Ronquist and Ågren 1974) In this paper it was reported that endogenous protein kinase of the surface of intact Ehrlich cells maintained in an isotonic suspension could catalyze the transfer of the terminal phosphoryl group of ATP into an acceptor protein in the external medium Phosvitin and histone were used as acceptor proteins The negatively charged phosvitin was about 20 times as effective as the positively charged histone molecules with respect to phosphorylation

It was therefore assumed that Ehrlich cells free in suspension may phosphorylate each other where the surface protein kinase of one single cell catalyzes the transfer of phosphoryl groups to the acceptor protein of another cell in the presence of (γ-^{32}P)ATP in the suspending medium Endogenous acceptor protein at the cell surface has been shown to be present at the surface of tumor cells (Ågren and Ronquist 1971 Kinzel and Mueller 1973)

The aim of the present paper is to extend the work with Ehrlich cells also to comprise glia and glioma cells in culture These cells represent also normal cells which offer comparison with the malignant glioma cells Furthermore these cells are not free in suspension but are grown in plastic Petri dishes thus constituting a monocellular layer with the cells attached to the dishes throughout the experiment (Ågren *et al* 1971 1974)

Table I illustrates the presence of protein kinase at the surface of intact glia and glioma cells In accordance with the results obtained from the experiments with Ehrlich cells phosvitin is more effective as an extrinsic acceptor protein than histone Furthermore as was the case with Ehrlich cells cyclic AMP is not stimulating the phosphorylation of phosvitin

TABLE I Incorporation of terminal phosphoryl group of (γ P)ATP into phosphoryl serine(SerP) phosphorylthreonine(ThrP) and phosphopeptides of acceptor proteins of glia and glioma cells in culture

		SerP	ThrP	phosphopeptides
1	histone glia	0 159	0 0230	0 028
2	histone glia + cycl AMP	0 295	0 044	0 050
3	histone glioma – cycl AMP	0 147	0 023	0 062
4	phosvitin glia	1 270	0 850	0 349
5	phosvitin glia + cycl AMP	1 203	0 137	0 147
6	phosvitin glioma	0 970	0 349	0 115
7	phosvitin glioma + cycl AMP	0 963	0 539	0 134

The results given are phosphoryl groups in pmol/1 × 10⁴ cells min tranferred into acceptor protein at 37 C

Surprisingly the glia cells had a higher protein kinase activity than the malignant glioma cells as compared on a cell count basis It is necessary to keep in mind however that the total cell volume of one million glioma cells is about 72 per cent of that of glia cells (Ågren *et al* 1974)

On the other hand the surface area of glioma cells is estimated to be larger than that of glia cells (Ponten 1974) It can therefore be assumed that the protein kinase at the surface of the cell is decreased during the transformation of normal glia cells into malignant cells This in accordance with the surface located ATPase activity which likewise shows a similar decrease (Ågren *et al* 1971)

In a recent paper (Ronquist and Ågren 1974) the possibility of a mechanism for intercellular regulatory function was claimed to be exerted by the cell to cell phosphorylation This model was based upon experiments with Ehrlich cell which represent an experimental system of free single cells in suspension The present results suggest that such a model is also valid for cells constituting a mono-layer and attached to the plastic dishes It is interesting to note that such cells also can directly interact with each other Therefore the model given might also be valid for cells organized in a parenchyma tissue

We thank prof J Ponten Uppsala, for his generous gift of the cells used in this work We are also indebted to Ing S Eklund Miss H Grundberg and Mr C Wernstedt for skilful technical assistance This investigation was supported by a grant from the Swedish Medical Research Council (project B74 13X 228 10C)

References

ÅGREN G and G RONQUIST Isolation of ³²P labelled phosphorylserine from Ehrlich mouse ascites tumour cells suspended in an isotonic medium containing ³²P labelled adenosine triphosphate *Acta phys ol scand* 1970 79 125—128

ÅGREN G and G RONQUIST Isolation of ³²P labelled phosphorylserine and phosphorylthreonine from Ehrlich mouse ascites tumour cells suspended in a medium containing ³²P labelled nucleoside triphosphate or inorganic pyrophosphate *Acta* [illegible] 25 2931—2934

AGREN G and G RONQUIST Isolation of ^{32}P labelled phosphorylserine and phosphoryl threonine from Ehrlich mouse ascites tumor cells suspended in different isotonic media, containing ^{32}P labelled adenosine triphosphate *Acta chem scand* 1974 In press

AGREN G J PONTEN G RONQUIST and B WESTERMARK Demonstration of an ATPase at the cell surface of intact normal and neoplastic human cells in culture *J cell Physiol* 1971 *78* 171—176

AGREN G J PONTEN G RONQUIST and B WESTERMARK Nucleoside Diphosphate kinase at the cell surface of neoplastic human cells in culture *J cell Physiol 1974 83* 91—101

KINZEL V and G MUELLER Phosphorylation of surface proteins of Hela cells using an exogeneous protein kinase and (γ ^{32}P)ATP *Biochim biophys Acta* (Amst.) 1973 *322* 337—351

LEMAY A M DESCENES S LEMATRE S POIRIER G POULIN and F LABRIE Phosphorylation of adenophyseal plasma membranes and properties of associated protein kinase *J biol Chem* 1974 *249* 323—328

PONTEN J Personal communication 1974

RONQUIST G and G AGREN Isolation of ^{32}P labelled phosphorylthreonine from Ehrlich mouse ascites tumour cells suspended in an isotonic medium containing ^{32}P labelled adenosine triphosphate *Acta chem scand* 1970 *24* 723—729

RONQUIST G and G AGREN (^{32}P)Phosphoryl transfer by endogeneous protein kinase at the Ehrlich cell surface into extrinsic acceptor proteins *Upsala J Med Sci* 1974 *79* 138—147

SCHLATZ L and C W MARINETTI Protein kinase mediated phosphorylation of the rat liver plasma membrane *Biochem biophys Res Commun* 1971 *45* 51—56

Acta physiol scand 1974 92 433—439

From the Institute of Physiology and Medical Biophysics Biomedical Center
University of Uppsala, Sweden

Capillary Permeability to Glucose and Raffinose Studied with Single-Injection Technique

By

Bo Aberg

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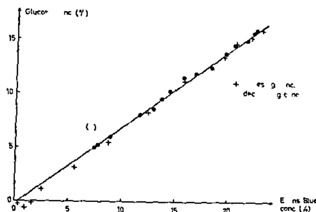
Abstract

Aberg B *Capillary permeability to glucose and raffinose studied with single injection technique* Acta physiol scand 1974 *92* 433—439

In a series of single inject on experiments on continuously weighed isolated cats hind legs the extractions of glucose and raffinose in pair were calculated The extraction was calculated by using albumin tagged with Evans blue as a reference The reference was found to exchange with the tissue and after a correction for this loss from the bolus the extraction ratio raffinose over glucose ranged between 0 681 –0 764 This was slightly above the ratio of the free diffusion coefficients (D raffinose/D glucose = 0 645) and much above the ratio of the correspond ng restricted d ffusion coeff cients (0 42—0 44 assuming an average pore rad us of 4 nm)

The passage of uncharged molecules across the capillary wall has been much dis cussed in the litterature and different models have been used to describe the phenomenon The earliest models of Starling (1896) and Landis (1927) considered ultrafiltration transport to be predominant Pappenheimer (*cf* Pappenheimer 1954) uggested a model for transcapillary transport mainly based on diffusional transport through homogenous pores (restricted diffusion) This model was extended by Grotte (1956) to a 2 pore model the larger pores being responsible for permeation of the protein moleclues As an alternative to transport through large pores Wınne (1965) in his calculations preferred pinocytosis to be the transport mechanism for the large molecules based on findings by Palade (*cf* Palade and Bruns 1968) Parallel to the 2 pore model a diffusion model without restriction was developed (Chinard *et al* 1955 Crone 1963)

In order to study capillary permeability two main investigative methods have been used Grotte (1956) and Renkin (1959) infused test substances Grotte in one large bolus with renal vessels occluded and Renkin with a continuous infusion When the blood concentration of the test substance or the test substances had reached a constant level samples were taken from the blod or from blood and lymp



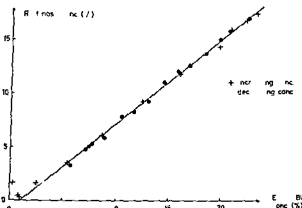


Fig 1 An example of the result obtained from a single injection experiment Note that the ratio of the effluent concentration of the diffusible substance to that of the reference fall on straight lines during the whole sampled effluent curve The extractions are calculated as 1 – (the angle of inclination of the bet fitted straight lines)

in order to calculate the transendothelial transport rate This method (with modifications) has been called the continuous infusion method (*cf* Introduction to Alfred Benzon Symposium II 1970) but as the technique varied and did not always involve continuous infusions it might be better to call it the *constant concentration method*

The Chinard Crone method is often called the *single injection technique* A bolus containing test and reference solutes is injected into the artery the reference being large enough not to pass through the blood tissue barrier From the organ to be studied samples are taken from a vein and analysed for their contents of the test and the reference substances The *extraction* can be determined by a comparison of the venous effluent concentrations of the diffusible and the reference substance As the main purpose of this technique and its variations (see Crone 1970) is to study the transendothelial transport from an intracapillary concentration pulse the *transient concentration method* might be a good common name for these method

Results from constant concentration experiments generally indicated that the

TABLE Ia Results from single injection experiments on the isolated hind legs of cats Extractions (E) calculated according to the regression method for raffinose and glucose (subscript r and g) the mean values are given ±S E

Exp no 1–13	E_r	E_g	E-quotient
Mean	0 208 ± 015	0 311 ± 019	0 678 ± 038

TABLE Ib The extractions and extraction quotients from Table Ia corrected for the loss of the albumin in each experiment The corrected extraction is marked with an asterisk

	E-quot	% alb loss / 100 g		E*-quotient
Mean	0 678 ± 038 (n=13)	1 6 ± 4	(n=13)	0 705 ± 037 (n=15)

transport across capillary walls is sterically restricted and the results from transient concentration experiments generally showed a transport proportional to free diffusion coefficients As it is hard to obtain an accurate transport ratio for 2 well defined substances with the latter method from the litterature because of the large variability this study was undertaken in order to obtain a transport ratio as exact as possible suitable for further studies

Methods

Experimental methods Single injection experiments were performed on isolated continuously weighed hind legs of cats The leg weight was held constant during the experiment (no net filtration) A full description of the method has been presented previously (Åberg and Lindahl 1973)

Analytical methods Parts of the venous outflow samples—without any treatment except dilution—were analyzed spectrophotometrically at 610 nm for Evans blue The rest of the samples were deproteinized with buffered perchloric acid and analysed for their content of raffinose in a spectrophotometrically recorded Seliwanoff reaction (Roe *et al* 1949) and glucose by a glucose-oxidase method (Åberg 1967)

Calculations Extractions were calculated according to a regression method This means that the venous effluent concentration of the diffusible test solutes (c_{diff}) was plotted against the concentration of the reference solute (c_{ref}) in each sample and E was calculated as

$$E = 1 - \Delta c_{diff} / \Delta c_{ref} \quad (5)$$

where $\Delta c_{diff} / \Delta c_{ref}$ was calculated from the best fitted straight line A prerequisite of this method is that the slope is close to a straight line which was always the case (Fig 1)

Results

The results from a series of experiments are listed in Table Ia From these data it can be seen that the extraction of raffinose/extraction of glucose (E_r/E_g) amounts to 0 678 ± 0 038 (S E) This rather large variation includes effect of variations in blood flow velocity as well as the individual variation of permeability between

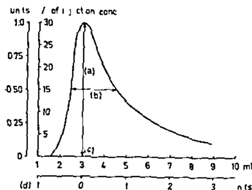


Fig 2 The normalization procedure All concentration values obtained from the venous samples are divided by their respective concentration in the injection solution and by the peak concentration of Evans blue labelled albumin (a) The accumulated outflow volumes are subtracted by the peak volume (c) and divided by the half peak width (b) Thus two dimensionless scales are obtained (to the left of an below the ordinary scales) These normalized values are used to correct for differing blood flow rates

preparations Bassingthwaighte *et al* (1966) showed that a plasma indicator was dispersed in the vessels its concentration along the vessel forming a lagged normal density curve They showed in experiments and calculations on the human leg that the mean transit volume V according to Stewart Hamilton of a vascular system was related to blood flow Q and mean transit time t as

$$V = Q_1 \; t_1 = Q_2 \; t_2 \tag{6}$$

In order to circumvent the effect of differing blood flow velocities the effluent concentration curves of the diffusible substances were normalized to that of albumin for each experiment in the following manner

Assume a venous effluent concentration curve of albumin as in Fig 2

The peak albumin concentration (a) is for example 30 % In order to obtain a dimensionless normalized concentration scale all concentrations are divided by this value The resulting albumin peak concentration will be 1 unit

Half albumin peak concentration (b) is 15 % at the outflow volumes 2 5 and 4 5 ml The distance between them is 2 ml

The peak appears at the outflow volume of 3 ml (c)

A normalized dimensionless volume scale (d) is obtained by subtracting 3 ml *(from (c))* from the accumulated outflow volume for each sample and dividing the rest by 2 *ml (from* (b)) Then the albumin peak volume is zero and the accumulated outflow volume unit 1 corresponds to the width of the half peak albumin concentration As the venous outflow rate is constant during the sampling the normalized volume scale also is a normalized time scale

The concentration values obtained by this normalization procedure (2 sets of 276 data points) were again calculated for extractions according to the regression procedure ($E = 1 - \Delta c_d / \Delta c$) and within the 99 % confidence interval (correlation calculated according to Bartlett 1949) The mean quotient of extractions of raffinose over glucose found by division and appropriate correction of the intervals ranged from 0 638 to 0 716 The quotient of free diffusion coefficients of 0 645 thus falls well within this range but the ratio of the restricted diffusion coefficients calculated for a pore radius of 4 nm (0 42—0 44 according to *Pappenheimer* (1953)) is much beyond the lower 99 % confidence limit

The calculations however heavily relies on the assumption that albumin was an ideal reference (i e it was not extracted from the blood) Analysis of the content of the venous effluent showed that albumin was lost from the capillary to some extent In order to calculate this albumin loss (although not very accurately) the venous recovery was determined in all the experiments by integration of the venous outflow curve of the albumin concentration A summary of such calculations on the present material is shown in Table Ib and the 99 % confidence interval for albumin loss is (0 96 7 02)

The extractions of glucose and raffinose were corrected for the albumin loss in the following manner

$$E^* = 1 - \frac{1-E}{f} \tag{7}$$

where E^* is the corrected correlation extraction and $f = (1 + \text{the fraction of albumin loss})$

The 99 % confidence interval for the experimentally obtained data were

$$1-E_r = 0\ 833 - 0\ 797$$
$$1-E_g = 0\ 750 - 0\ 703$$

Now the extrenes of the extraction quotients can be calculated

$$\frac{E^*_r}{E^*_g} = \frac{1 - \frac{0\ 833}{1\ 0096}}{1 - \frac{0\ 750}{1\ 0096}} \text{ to } \frac{1 - \frac{0\ 797}{1\ 0702}}{1 - \frac{0\ 703}{1\ 0702}}$$

$$\text{or } \frac{E^*_r}{E^*_g} = 0\ 681 \text{ to } 0\ 744 \text{ with a mean value of } 0\ 713$$

The result of this normalization was a slightly increased mean value but it still differs very little from the earlier findings that extraction quotients are comparable to quotients of free diffusion coefficients and that they are not compatible with the restricted diffusion coefficient rations

Discussion

Validity of the steady state concept The main critizism of the single injection technique and of the evaluation of extractions is If extractions are calculated from the major part of the venous effluent curve the extraction is not a measure of the unidirectional solute transport from blood to tissue but affected by back transport from tissue to blood If on the other hand only the first parts of the effluent curves are used in the extraction calculations only the extractions from the exchange vessels with the shortest transit times are investigated In the experiments reported here extractions were calculated from the major part of the venous effluent curve

This could be done since in each experiment the plot of the venous effluent concentrations of reference versus diffusible substance fall on straight lines. This preparation thus behaves as if the extractions of *raffinose and glucose were close to a diffusion limited transport* which indicates that the vascular bed investigated was well fitted for extraction experiments with the substances chosen. It should be noted that in this case, the extractions calculated with the regression method differs very little from extractions calculated with other methods.

The albumin loss. The extraction of raffinose and glucose was calculated with albumin as the reference substance. Albumin was found to exchange with the tissue. Some *loss could be expected since it is well known that albumin and molecules of* that size exchanges with the tissues. Normally, however, the albumin loss during single injection experiments can be neglected in most tissues. The rather large losses in the experiments reported here indicates an abnormally high permeability to large molecules.

Concluding remarks. In the present experiments the extraction ratio for raffinose and glucose was much higher than expected from the restricted diffusion coefficients. This result *is in* good agreement with previous observations in experiments *with* similar molecules (*cf* Kampp 1970). One explanation might be that ultrafiltration through the capillary pores has an effect on the outflux of these molecules.

In an earlier publication (Åberg and Hagglund 1974) the effect of convection on diffusion through a filtration pore was studied in model experiments. An extension of this model will be discussed in a forthcoming paper.

My thanks are due to my colleagues at the Institute of Physiology and Medical Biophysics for valuable discussions. I also wish to express my gratitude to Miss Carin Ferner, Mrs Ulla Britta Berg and Mrs Helena Billander for very skilful technical assistance. *This work was* supported by grants from the Medical Faculty of the University of Uppsala, the Swedish Medical Research Council (Project No 14X 629) and the National Institutes *of Health* (Grant No R 01 HE 12960-08).

References

ALFRED BENZON Symposium II. *Capillary Permeability*. Ed C Crone and N A Lassen. Munksgaard, København 1970.

ÅBERG B. Interference of light on the determination of low glucose concentrations with glucose oxidase. *Acta physiol scand* 1967 71 186—193.

ÅBERG B and J V HAGGLUND. A convection-diffusion model of capillary permeability with reference to single injection experiments. *Upps J Med Sci* 1974 79 —1?

ÅBERG B and S O LINDAHL. An *in vitro* method of investigating the rates of transcapillary exchange and net filtration by single injection technique. *Upps J Med Sc* 1973 78 145—149.

BARTLETT M S. Fitting a straight line when both variables are subjected to error. *Biometrics* 1949 5 207—212.

BENCHTHWAITE J B, F H ACKERMAN and E H WOOD. Applications of the lagged normal density curve as a model for arterial dilution curves. *Circula Res* 1966 18 39?—415.

CHINARD F P, G J VOSBURGH and T ENNS. Transcapillary exchange of water and of other substances in certain organs of the dog. *Amer J Physiol* 1955 183 221—234.

CRONE C. The permeability of capillaries in various organs as determined by use of the "indicator diffusion" method. *Acta physiol scand* 1963 58 292—305.

CRONE C. Capillary permeability—techniques and problems. In *Alfred Benzon Symposium II* (see first ref.) 1970 15—31.

GROTTE G Passage of dextran molecules across the blood lymph barrier *Acta chir scand* 1956 Suppl 211

KAMPP M The diffusion permeability of some artificial and of some capillary membranes related to the free diffusion coefficients of the probe molecules In Alfred Benson Symposium II (see first ref) 1970 163—170

LANDIS E M Micro-injection studies of capillary permeability *Amer J Physiol* 1927 *82* 217—238

PALADE G E and R R BRUNS Structural modulations af plasmalemmal vesicles *J cell. Biol* 1968 *37* 633—649

PAPPENHEIMER J R Passage of molecules through capillary walls *Physiol Rev* 1953 *33* 387—423

PAPPENHEIMER J R Ultrafiltration and diffusion through biological membranes *Annual Lecture* 1954 No I Bethesda, Md Nat. Inst Health

RENKIN E M Transport of potassium-42 from blood to tissue in isolated mammalian skeletal muscles *Amer J Physiol* 1959 *197* 1205—1210

ROE J H J H EPSTEIN and N P GOLDSTEIN A photometric method for the determination of inulin in plasma and urine *J biol Chem* 1949 *78* 839—845

STARLING E H On the absorption of fluids from the connective tissue spaces *J Physiol* (Lond) 1896 *19* 312—326

WINNE D Die Capillarenpermeabilitat hochmolekularer Substanzen *Pflugers Arch ges Physiol* 1965 *283* 119—126

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From the Biophysical Laboratory and the Departments of Prosthetic Dentistry Oral Roentgenology and Pathology University of Umea Sweden

The Variation with Age of Tissue Zinc Concentrations in Albino Rats Determined by Atomic Absorption Spectrophotometry

By

Bo Bergman, Rolf Sjostrom and Kenneth R Wing

Received 5 April 1974

Abstract

Bergman B R Sjostrom and K R Wing *The variation with age of tissue zinc concentrations in albino rats determined by atomic absorption spectrophotometry* Acta physiol scand *1974 92* 440—450

An atomic absorption spectrophotometric method for the determination of zinc concentrations in biological tissue samples has been refined All samples are ashed for 16 hours at 550 C and each determination on a mineralized tissue sample is corrected for the absorption due to the high calcium concentration by subtracting a further absorption measurement at a second wavelength The method is without detectable systematic error except for 15—20 % losses of zinc from serum samples and the random error is less than 2 % The method was compared with neutron activation analysis on duplicate tissue ash samples and the agreement of the determinations was very good The method was used to determine the zinc concentrations in samples of serum heart and selected viscera teeth and bones from albino rats at various ages during growth Of these only the zinc concentration in heart is stable In pancreas and incisor crowns the concentrations are correlated to the serum concentration and those in the bone samples are correlated to the total mineral (ash) content in each The ash weight zinc concentrations in bones the fresh weight concentrations normalized to the total mineral content are correlated to the serum zinc concentrations

Key words
Zinc bone and bones teeth rats atomic absorption spectrophotometry

Introduction

As zinc is to a large extent not free as an ion in the body but bound to macromolecules in tissues and body fluids (Parisi and Vallee 1969 Underwood 1971) and as zinc is essential to the activity of several enzymes and is probably important to the synthesis of RNA DNA and protein (Prasad *et al* 1971 Mills *et al* 1969) to the synthesis storage and release of insulin (Falkmer 1971) and to the process of mineralization in bone (Follis *et al* 1941 Bergman *et al* 1972) variations in the zinc concentrations in the body may both reflect as well as be the cause of changes in metabolism The zinc concentrations in bones and teeth may not be stable

throughout life (Taylor 1961 Bergman 1970 a) and animals fed zinc-deficient diets lose zinc from some tissues particularly bones and teeth with resultant impairment of normal function growth and development (Mills *et al* 1969 Oberleas and Prasad 1969 Bergman *et al* 1972) The nature of the control the body may have over tissue zinc concentrations and the ability of the body to mobilize zinc reserves particularly the large quantities of zinc in bone during periods of low dietary intake are uncertain (Miller 1969 Hurley 1969 Bergman 1970 b c)

A series of investigations was initiated in order to obtain more information on the nature of the turnover of zinc in the serum viscera heart teeth and bones of rats fed conventional pellet and zinc-deficient diets In the first of these the variations in the zinc concentrations in growing rats were studied The results of the analyses of the turnover of ^{65}Zn in rats fed conventional and zinc-deficient diets are presented in subsequent articles (Wing 1974 Bergman and Wing 1974)

A method for the determination of zinc concentrations was sought to replace neutron activation analysis which has earlier been used in our laboratory (Bergman 1970 a c) but is costly cumbersome and incompatible with tracer studies using ^{65}Zn An atomic absorption spectrophotometric method was refined for the determination of zinc concentrations even in tissues with high mineral salt concentrations and after being subjected to a series of method error studies was used in the present investigations

Materials and Methods

Albino rats of the Sprague Dawley strain were used in the present study The rats were lightly anesthetized with ether and killed by decapitation The procedure used in taking the samples has been described earlier (Bergman 1970 a) The following samples were taken serum kidney pancreas spleen liver (median lobe) heart incisors (pulpless crowns) mandibular condyl mandibular bone (ramus and part of corpus) tibia epiphysis and tibia diaphysis (bone marrow removed) In order to avoid contamination from metal instruments only polyethylene or polyethylene-covered instruments were allowed to come in direct contact with the samples The samples were collected in weighed Pyrex tubes which had been washed in conc HCl The fresh sample weights were recorded immediately

The samples were dried in an oven for 4 to 6 h at 100 C and then overnight at 150 C The samples were transferred to a muffle oven and the temperature slowly raised and then maintained at 550 C for a second night. When the samples had cooled to room temperature the ash weights were recorded and the ash dissolved in 0.5 ml 10.5 % HCl overnight All samples were then diluted with 2.0 ml 3x-distilled water and further diluted with 2.2 % HCl as needed The zinc concentrations were determined using a Unicam SP90 spectrophotometer at 213.9 nm with a zinc lamp All samples were aspirated twice Reference samples of zinc in 2.2 % HCl were used

Calcium concentrations of the order of those in the mineralized tissue samples cause significant absorption at 213.9 nm which results in overestimation of the zinc concentrations by as much as 10 % or more The absorption for calcium at 210 nm with the zinc lamp is equal to that at 213.9 nm while none of the zinc references shows any detectable absorption at 210 nm Thus the total absorption for the sample at 213.9 nm can be corrected for the absorption due to calcium by subtracting the absorption for the sample at 210 nm Samples were prepared containing zinc and various concentrations of *pro analysi* tricalcium phosphate (Merck Darmstadt West Germany) These samples were dissolved as above and the zinc concentrations determined by this method The recovery of the zinc is 99—100 % with calcium concentrations as high as 3000 times the zinc concentration ($\mu g/ml$) The random error in the method was estimated on 10 duplicate dissolved tissue ash samples for each of the eleven samples and is 2 % for serum $\geqslant 0.5$ ml serum the error is larger for smaller samples) 1 % for viscera and heart and 1.5 % for the mineralized tissues

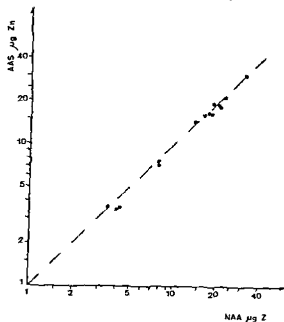


Fig 1 A scatter diagram of the total zinc in each of 33 serum and tissue ash samples as determined by atomic absorption spectrophotometry (ordinate) and neutron activation analysis (abscissa) N.B both axes are logarithmic The 45 line representing perfect agreement of the methods has been drawn as a reference

A comparison was made between the zinc concentrations determined by neutron activation analysis (NAA) and those determined by atomic absorption spectrophotometry (AAS) on duplicate dissolved tissue ash samples All 11 samples were represented 33 duplicate determinations were made in all The duplicate sample weights and the original sample weight were used to calculate the 2 determinations of the total zinc in the original samples

The results of this comparison are presented in Fig 1 The mean relative difference between the determinations $100\ \overline{(AAS\ NAA)/NAA}$ is $-3\ 1$ % (S.D $-12\ 7$ %) A t test of the mean relative difference demonstrates that it may not be a real difference ($t = 1\ 40$ $p > 0\ 05$) The combined standard deviation of the two determinations is 9 5 % and appears to apply to the entire range of the zinc concentrations (Fig 1)

For the study of the variation with age of the zinc concentrations in serum heart viscera, teeth and bones 32 weanling female rats were at random assigned to 1 of 4 cages thus each cage contained 8 rats at the beginning of the experiment The cages were made of acrylic resin with stainless steel covers The rats had free access to ordinary tap water (< 0 05 µg zinc/g) and a conventional pellet diet (Anticimex 210 Anticimex Norrviken Stockholm 70—75 µg zinc/g) 4 rats one taken at random from each cage were killed at 3 4 6 8 12 16 24 and 32 weeks of age and the samples taken and the zinc concentrations determined using atomic absorption spectrophotometry as described above An additional group of six weanling male rats housed in a single cage were fed the same diet and killed two at a time at 3 6 and 12 weeks of age

The calculations of the concentrations and their means and standard deviations as well as the correlation and variance analyses were performed using an electronic computer (Control Data 3200 Umea datacentral) or a programmable calculator (Canon Canola 167P) with standard programs Correlation coefficients were derived both for the group means and for the individuals in the groups taken two consecutive groups at a time (e g 3 and 4 week old groups together $n = 8$) If the null hypothesis that the 4 group correlation coefficients came from the same population was not rejected the 4 were pooled to form a common coefficient for the 32 rats (Snedecor and Cochran 1967) In the presentation of the results of statistical tests the following levels of significance are used

$p \leqslant 0\ 001$ highly significant **
$0\ 001 < p \leqslant 0\ 01$ significant**
$0\ 01 < p \leqslant 0\ 05$ nearly significant*
$0\ 05 < p$ not significant

where p is the probability of incorrectly rejecting the null hypothesis

TABLE I The fresh weight zinc concentrations in ppm ($\mu g/g$) in the 11 samples from female albino rats killed in groups of four at 3 4 6 8 12 16 24 and 32 weeks of age The ash weight/fresh weight ratios and the ash weight zinc concentrations in ppm in the mineral ized tissue samples are also gi en The means are those for all 32 rats and the standard deviations are those for the individual age groups (within) and all 32 rats (total) obtained in the analysis of variance on the eight age groups the results of which are presented under F with the levels of significance

	$\bar{x}$	S D with n	S D total	F
Zn ppm f esh wei ht				
serum	1 49	0 ?2	0 28	4 0?*
kidney	24 1	1 3	2 1	8 64***
pancreas	22 6	2 9	4 7	7 75 **
spleen	22 6	0 7	1 0	6 16***
liver	29 1	1 9	3 6	13 00**
heart	17 7	0 8	0 9	2 ?1
incisors	96 7	5 9	1_ 0	14 5? *
mand condyle	139 1	13 7	36 4	27 99
mand bone	152 9	10)	25 1	19 94
tib piphysis	125 8	11 9	58 4	10? 64* *
tib d aphysis	175 9	11 4	23 3	14 89* *
Ash wei ht f h wei ht				
incisors	0 ?75	0 019	0 033	9 19***
mand condyle	0 314	0 028	0 106	59 38***
mand bone	0 461	0 0?2	0 111	114 16***
tib epiphysis	0 244	0 017	0 107	165 80***
tib d aphys s	0 545	0 018	0 080	76 56 **
Zn ppm ash we ght				
incisors	143 9'	7 3	22 3	36 81*
mand condyle	478 7	37 3	148 3	64 26* *
mand bone	345 0	16 3	71 9	82 39***
tib ep physis	519 8	34 2	56 5	8 65 *
t b diaphysis	326 6	23 4	40 8	9 97*

n = 30 n = 31 3 samples w re spoiled in preparat on

Results

The results of the determinations of the fresh weight zinc concentrations in the eleven samples from the female albino rats at ages from 3 to 32 weeks are presented as ppm ($\mu g/g$) fresh weight in Table I and Fig 2 Approximately 75 % of the coefficients of variation (100 S D/$\bar{x}$) for the individual age groups which are also equal to two standard errors of the mean (n = 4) are less than 10 % and they average 8 % The highest coefficients of variation for the individual age groups are those for the zinc concentrations in serum and pancreas and the lowest are for tho e in spleen

The zinc concentration in serum i the lowest of the eleven samples The mean serum concentrations vary considerably among the age groups and the analysis of variance is significant The coefficient of variation for the zinc concentrations in serum is lowest for the 12 week old group which has the lowest concentration The zinc concentrations in viscera and heart are 10 to 30 times that in serum The zinc

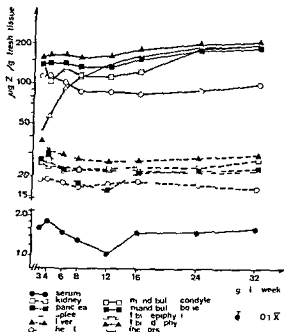


Fig 2 The concentrations of zinc expressed as ppm (μg/g) fresh weight in serum and selected tissues from female albino rats *N B* log scale for concentration Each point represents the mean concentration in samples from 4 rats The bar represents a coefficient of variation (100 SD/x̄) of 10 %

concentration in pancreas varies greatly and those in the remaining viscera vary moderately among the age groups The concentration in heart is quite stable The analyses of variance are highly significant for the concentrations in the viscera and not significant for that in heart The group mean concentrations in pancreas are highly significantly positively correlated to those in serum (r = 0 96***) The pooled group correlation is positive and highly significant (r = 0 64***) The mean zinc concentrations in kidney spleen liver and heart are not significantly correlated to those in serum (r = 0 50—0 60)

The mineralized tissues exhibit fresh weight zinc concentrations 30 to 150 times that in serum The concentration in tibia epiphysis changes most during growth being the lowest at three weeks and undergoing a 5 fold increase from 3 to 32 weeks The remaining bones show some fluctuation in concentration in the younger rats but from 12 to 24 weeks of age they show moderate steady increases In the adult rats the zinc concentrations in the bones are very nearly equal The zinc in incisor crowns decreases slightly from 3 to 8 weeks of age and thereafter remains very nearly constant The results of the analyses of variance on the fresh weight zinc concentrations in the 5 mineralized tissue samples are highly significant Of the mean fresh weight zinc concentrations in mineralized tissues those in incisors are alone in being nearly significantly positively correlated to those in serum (r = 0 75*) The pooled group correlation is not significant (r = 0 25)

The ash weight/fresh weight ratios for the mineralized tissue samples are presented in Table I and Fig 3 The coefficients of variation for the individual age groups average 5 % and are less than or equal to 10 % in all but a few of the samples at

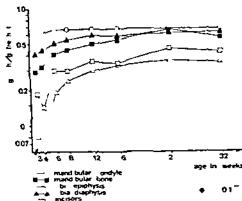


Fig 3

Fig 3 The ratios of the ash weight to fresh tissue weight in 5 mineralized tissues from female albino rats N.B log scale for ratio Each point represents the mean ratio in samples from four rats The bar represents a coefficient of variation (100 S.D/x̄) of 10%

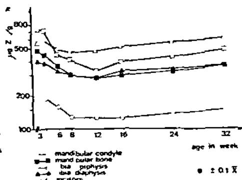


Fig 4

Fig 4 The concentrations of zinc in 5 mineralized tissues from female albino rats expressed as ppm (μg/g) ash weight. N.B log scale for concentration Each point represents the mean concentration in samples from 4 rats The bar represents a coefficient of variation (100 S D/x̄) of 10%

a few ages The zinc concentrations in these tissues have also been calculated on the basis of the ash weights (μg/g) of the individual samples and are presented in Table I and Fig 4 The coefficients of variation for the individual age groups average 6% They are with few exceptions less than 10% and in most cases are less than those for the zinc concentrations based on the fresh weights for the corresponding organ and age

Of the mineralized tissues sampled the incisors contain most mineral At 3 weeks the cortical bone in the tibia diaphysis and predominantly cortical bone of the mandible contain less mineral and the endochondral bone of the condyle and the calcifying cartilage of the tibia epiphysis contain least mineral From 3 to 8 weeks further mineralization in the bones proceeds rapidly and then slows considerably until in the adults the ash content is relatively constant The analyses of variance are significant or highly significant for the ratios in all 5 samples The group mean fresh weight zinc concentrations in the tibia epiphysis are highly significantly positively correlated to the respective mean total mineral (ash) concentrations (0 93***) while those in the remaining bones are nearly significantly positively correlated (r = 0 72*—0 77*) and those in incisors are not positively correlated (r = −0 74) to the respective mean mineral concentrations The pooled group correlation coefficient is highly significant for the tibia epiphysis (r = 0 87***) and nearly significant and positive for the incisors (r = 0 48*)

Expressing the zinc concentration in bones and teeth as ppm ash weight produces a pattern which is quite different from that of the concentrations in ppm fresh weight From 3 to 8 weeks of age the zinc concentrations decrease to

TABLE II The mean fresh weight zinc concentrations in ppm (μg/g) in the 11 samples from male albino rats killed in groups of two at 3 6 and 12 weeks of age The mean ash weight/fresh weight ratios and mean ash weight zinc concentrations in the mineralized tissue samples are also given

	3 weeks $\bar{x}$	6 weeks $\bar{x}$	12 weeks $\bar{x}$
Zn ppm fresh weight			
serum	1 42	1 49	1 66
kidney	25 0	24 0	25 5
pancreas	29 8	25 0	20 1
spleen	22 6	21 9	22 5
liver	34 3	33 4	28 1
heart	18 1	17 3	18 1
incisors	101 9	93 6	101 0
mand condyle	89 0	91 9	145 8
mand bone	142 4	139 0	[illegible]
tib epiphysis	37 4	81 1	[illegible]
tib diaphysis	173 1	168 2	[illegible]
Ash weight/fresh weight			
incisors	0 668	0 701	0 656
mand condyle	0 176	0 219	0 367
mand bone	0 336	0 446	0 530
tib epiphysis	0 077	0 178	0 281
tib diaphysis	0 449	0 506	0 540
Zn ppm ash weight			
incisors	152 6	133 6	154 0
mand condyle	507	420	398
mand bone	424	312	339
tib epiphysis	486	458	514
tib diaphysis	386	332	373

degrees in the 5 tissues stabilize during the next eight weeks and increase somewhat from 16 through 32 weeks The analysis of variance is significant or highly significant for the concentrations in each of the 5 samples The group mean ash weight zinc concentrations in tibia diaphysis are significantly positively correlated to the fresh weight zinc concentrations in serum ($r = 0.84^{**}$) while those in the remaining bones and the incisors are nearly significantly positively correlated ($r = 0.72^{*}$—0.77^{*}) The pooled group correlation coefficients for these comparisons are nearly significant for the mandibular condyle and tibia epiphysis ($r = 0.51^{*}$ 0.53^{*})

The wet weight zinc concentrations in serum and the tissues of the 6 male rats are presented in Table II The average coefficient of variation for all samples and groups is 5 % In most samples the concentrations fall within the range $\bar{x} \pm 2$ SD for the corresponding samples from the females of the same age However the concentration in serum is not low at 12 weeks as it is in the females and while the concentrations in pancreas and liver decrease with increasing age the levels and rates of decline differ from those in the females The concentrations in kidney spleen and heart are quite stable and approximately equal to those in the females The fresh weight zinc concentrations in the bones increase with age and that in the

tibia epiphysis is directly related to the mineral content as it is in the females The zinc concentrations in the mineralized tissues of the males are generally lower at 6 weeks and higher at 12 weeks than those in the females The mandibular condyle mineralizes later and the tibia diaphysis earlier and the mandibular bone is at all ages through 12 weeks more mineralized than in the females The ash weight zinc concentrations decrease from 3 to 6 weeks in all 5 mineralized tissues and rise again by 12 weeks in all but the condyle They are less than those in females at 3 and 6 weeks except in tibia diaphysis and greater at 12 weeks These data were not subjected to further statistical analysis as they are too few

Discussion

The sources of both random and systematic error in the method have been investigated in detail In order to study the possible loss of zinc from the samples during preparation ^{65}Zn was used as a tracer for zinc and injected into four 3 week old and four 24 week old rats Two rats from each group were killed at one day and two at one week after the injections The samples were taken in the usual manner and their ^{65}Zn activities measured both before and after the drying ashing procedure described above No detectable ^{65}Zn was lost from the samples and none was retained in the small amount of insoluble ash which remained in some of the tubes after dissolution with 10 5 % HCl For the serum samples only approximately 15—20 % of the ^{65}Zn was retained in the Pyrex tubes This loss occurs most likely as the result of the etching of the glass at 550° C by the NaCl in the serum samples causing the glass to take up zinc (Gorsuch 1970) The zinc concentrations in serum should thus be multiplied by a factor of 1 2 if they are to be more accurate This error does not affect the results of the statistical analyses presented

Samples of sodium chloride sodium citrate and tricalcium phosphate containing undetectable amounts of zinc were dissolved or suspended in water to simulate the samples from rats and then dried ashed and dissolved in the same manner as the samples from rats No uptake of zinc by the samples from the Pyrex tubes could be detected Thus despite zinc s reported vaporization at temperatures below 500° C (Gorsuch 1970) and the closeness of the ashing temperature to that at which Pyrex glass begins to soften no systematic error due to the loss of zinc from the samples except for the loss of zinc from the serum samples to the glass tubes and no error due to the contamination of the samples with zinc from the tubes was discovered

Ashing of the samples reduces the total variation of the zinc concentrations in all samples in several samples to one third to one fourth of that obtained when the samples are not ashed but dissolved in conc HCl the samples of serum viscera and heart further being treated with 4 % trichloroacetic acid to precipitate protein As the coefficient of variation (100 S D /x) for the atomic absorption method is less than 2 % the greater portion of the variation in the data in Fig 1 may be attributable to the neutron activation method error which has generally been found to be 10 % (Soremark 1965)

The drying and ashing procedures cause no measurable change in the weight of the Pyrex tubes Further ashing of the mineralized tissue samples in platinum receptacles for 18 h at 850° C produces less than 1 % further reduction in the weight of the ash As the loss of significant amounts of the most common mineral salts in bones and teeth is not expected at temperatures up to 550° C (Gorsuch 1970) the ash weight/fresh weight ratios should be good approximations to the mineral contents The calcium content in the ash of the mineralized tissues was determined in a separate experiment and the concentrations were found to increase slightly during growth (average calcium incisors 36 % mandibular bone and tibia diaphysis 38 % mandibular condyle and tibia epiphysis 37 %)

The patterns of the variation of the zinc concentrations in the various tissues are more complex than those suggested by previous results (Taylor 1961 Alexander and Nusbaum 1962 Bergman 1970 a) The mean zinc concentrations in serum vary moderately with age and the coefficients of variation for the age groups are the highest of those in the 11 samples The serum concentration at 12 weeks is unusually low and has the lowest coefficient of variation While the serum concentration may decrease during the growth period in females rats the particularly low concentration at 12 weeks may have been caused by an interruption in the normal feeding schedule during the night prior to sacrifice of this group

Among the zinc concentrations in the viscera and heart that in pancreas is the most strongly correlated to the serum concentration and shows the greatest variation The pooled correlation is also strong indicating that the equilibration of zinc in pancreas with that in serum may occur quite rapidly As the pancreas is the organ responsible for most of the zinc excretion (Underwood 1971) the correlation of the zinc concentration to that in serum is perhaps to be excepted The concentrations in the remaining viscera also vary during growth the pattern generally being one of moderate loss during the early growth period but the correlations to the serum concentration are much weaker The concentration in heart is quite stable

Zinc deficient feeding studies (*e g* Bergman 1970 c) have shown the zinc concentrations in some of the viscera and heart to be nearly normal despite extremely low zinc intake This might indicate the presence of homeostatic control mechanisms for the maintenance of minimum zinc concentrations in these tissues (Bergman 1970 b c) However since the strength of the binding of zinc to enzymes in these tissues varies from enzyme to enzyme and tissue to tissue (Prasad and Oberleas 1971 Prasad *et al* 1971) findings which presumably apply to the binding of zinc by other macromolecules it is also possible that the differences among the serum viscera and heart with respect to the variations in the zinc concentrations are the result of variations in the strength of the binding of zinc rather than variations in the activities of control mechanisms Further discussion of this topic is to be found in a report of the turnover of ^{6}Zn in rats fed a zinc deficient diet (Bergman and Wing 1974)

The fresh weight zinc concentrations in the bone samples vary considerably during

growth and are strongly correlated to the mineral contents (ash weight/fresh weight) Expressing the zinc or mineral content of a bone or tooth in terms of its fresh weight is not fully satisfactory for several reasons In all mineralized tissues the mineral content directly affects the density Thus increased mineral content reduces the apparent fresh weight zinc concentration without loss of zinc and increases in the mineral content are less apparent the more mineralized a sample is Correlations between fresh weight zinc and mineral concentrations are distorted due to the effect of the mineral content on their common denominator

Perhaps the only true expressions for the zinc and mineral concentrations would be ones based on morphologically well defined tissue volumes which could be followed throughout growth While this would present many difficulties the fresh weight zinc and mineral concentrations in the teeth and bones can be recalculated on a per volume basis averaged over the entire sample using the ash weight/fresh weight ratios and the densities of the mineral organic material and fluids in these tissues The resulting curves for the bone samples would resemble those in Fig 2 and 3 except that as the mineral contents increase both the zinc concentrations and the ash weights per unit volume increase more than in the figures For the incisor crowns the early decrease in zinc content would be almost negligible while the increase in the mineral concentration would be more than that in the figure More important the strong positive correlations between the zinc and mineral concentrations in the bone samples would become even stronger and the negative correlation for the incisors would decrease

The relationship between the zinc and mineral concentrations is seen in the ash weight zinc concentrations in Fig 4 From 3 through 8 weeks of age or more the zinc concentrations normalized to the mineral contents decrease and in the adults increase slowly parallel to one another The ash weight zinc concentration is highest in the calcifying cartilage of the tibia epiphysis and least in the incisor crowns or the inverse of the percent mineralized tissue The strong correlations between the ash weight zinc concentrations and the serum concentration indicate that the fresh weight zinc concentrations in mineralized tissues reflect the general trend of the serum zinc concentration as well as both the organization of the mineral and the degree of mineralization As milk particularly colostrum provides significant amounts of zinc to weaning mice (Nishimura 1953) this could be the source of the high initial zinc concentrations in the serum pancreas liver and mineralized tissues which decrease after weaning Further discussion of this topic is to be found in a report of the turnover of ^{65}Zn in rats fed a zinc deficient diet with and without a zinc supplement (Bergman and Wing 1974

The zinc concentrations in the serum viscera and heart of the male rats are nearly the same as those in the females The concentrations in pancreas and liver decrease with increasing age as they do in the females while that in serum does not The zinc and mineral concentrations in the bones of the males follow patterns slightly different from those in the females which may reflect differences in growth patterns the males growing faster and continuing to grow for a longer time Howeve

the data confirm the general relationship of the zinc concentrations to the organiza tion of the mineral and the degree of mineralization seen in the females

This study was supported by a grant from the Swedish Medical Research Council (Project B73 24X 3342 02)

Note added in press The loss of zinc from serum samples to the Pyrex tubes is negligible if the ashing temperature is reduced to 500 C

References

ALEXANDER G V and R. E NUSBAUM Zinc in bone *Nature* (Lond) 1967 *193* 903

BERGMAN B Concentration of zinc in some hard and soft tissues of rat determin d by neutron activation analysis *Acta radiol Ther Phys Biol* 1970 a 9 420—432

BERGMAN B The distribution and concentration of zinc and the effect of zinc deficiency in the mammalian body *Odont Revy* 1970 b *21* Suppl. 20

BERGMAN B The zinc concentration in hard and soft tissues of the rat. The influence of zinc deficient feeding *Acta odont scand* 1970 c *28* 425—440

BERGMAN B U FRIBERG S LOHMANDER and T ÖBERG The importance of zinc to cell proliferation in endochodral growth sites in the white rat *Scand J dent Res* 1972 *80* 486—492

BERGMAN B and K. R. WING The turnover of ^{65}Zn in rats fed a zinc-deficient diet. *Acta physiol scand* 1974 *92* 451—464

FALKMER S Sulfhydryl compounds and heavy metals in islet morphology and metabolism *Proc VII Congr Internat Diab Fed Buenos Aires Aug 1970* Exc Med Internat. Congr Ser 1971 *231* 219—225

FOLLIS JR. R H H. G DAY and E V MCCOLLUM Histological studies of the tissues of rats fed a diet extremely low in zinc *J Nutr* 1941 *22* 223—227

GORSUCH T T The destruction of organic matter *Internat Ser Monogr analyt Chem* 1970 *39* Chapter 8 pp 53—84

HURLEY L S Zinc deficiency in the developing rat *Amer J clin Nutr* 1969 *22* 1332—1339

MILLER W J Absorption tissue distribution endogenous excretion and homeostatic control of zinc in ruminants *Amer J clin Nutr* 1969 22 1323—1331

MILLS C F J QUARTERMAN J K. CHESTERS R B WILLIAMS and A C DALGARNO Metabolic role of zinc *Amer J clin Nutr* 1969 *22* 1240—1249

NISHIMURA H Zinc deficiency in suckling mice deprived of colostrum *J Nutr* 1953 *49* 79—97

OBERLEAS D and A S PRASAD Growth as affected by zinc and protein nutrition *Amer J clin Nutr* 1969 *22* 1304—1314

PARISI A F and B L VALLEE Zinc metalloenzymes characteristics and significance in biology and medicine *Amer J clin Nutr* 1969 *22* 1222—1239

PRASAD A. S and D OBERLEAS Changes in activities of zinc-dependent enzymes in zinc deficient tissues of rats *J appl Physiol* 1971 *31* 842—846

PRASAD A S D OBERLEAS E R MILLER and R. W LUECKE Biochemical effects of zinc deficiency Changes in activities of zinc-dependent enzymes and ribonucleic acid and deoxyribonucleic acid content of tissues *J lab clin Med* 1971 *77* 144—157

SNEDECOR G W and W G COCHRAN *Statistical Methods* 6th ed Iowa State Univ Press Iowa City 1967 pp 185—188

SOREMARK R Micro element analysis by neutron activation In *Progress in Atomic Med* (ed Lawrence J H Grune & Stratton New York 1965 pp 54—77

TAYLOR D M Retention of zinc-65 in the bones of rats *Nature Lond*) 1961 *189* 932—933

UNDERWOOD E J *Trace elements in human and animal nutrition* 3rd ed Academic Press New York 1971 pp 208—252

WING K R A comparison of the turnover of ^{65}Zn and ^{85}Sr in growing rats *Acta rad ol Ther Phys Biol* 1974 In press

Acta physiol scand 1974 92 451—464

From the Biophysical Laboratory and the Department of Prosthetic Dentistry and Oral Roentgenology University of Umea Sweden

The Turnover of ^{65}Zn in Rats Fed a Zinc-Deficient Diet

By

Bo Bergman and Kenneth R Wing

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Abstract

Bergman B and K. R. Wing *The turnover of ^{65}Zn in rats fed a zinc deficient diet* Acta physiol scand 1974 92 451—464

The fresh weight zinc concentration in spleen is unchanged the concentrations in serum kidney pancreas liver and heart are reduced moderately and those in incisors and four bone samples are reduced markedly in rats fed a zinc-deficient diet (7 2 μg Zn/g) for six weeks compared to those in rats pair fed the same diet and given a 200 μg zinc supplement daily The ash weight zinc concentrations in mineralized tissue samples from both groups decrease rapidly and after three weeks on the diet assume relatively stable levels The retention of ^{65}Zn in the serum heart and viscera is higher in rats not given the supplement. The pattern is consistent with decreased turnover of zinc due to reduced intake and excretion The ^{65}Zn retention data from the bones and teeth were analyzed using the Bauer Carlsson Lindquist equation Reduced zinc intake causes a decrease in bones of the rate at which zinc is accreted most likely as a result of decreased accretion of bone as well as decreased availability of zinc The slowly exchanging zinc in incisors and cortical bone is markedly reduced while the rapidly exchanging zinc in bones is less affected

Key words Zinc metabolism zinc and zinc isotopes zinc deficiency bone and bones teeth

Earlier studies (Macapinlac *et al* 1966 Prasad *et al* 1967 Swenerton and Hurley 1968 Huber and Gershoff 1970 Bergman 1970 a) have demonstrated significant losses of zinc from bones in rats fed zinc-deficient diets while the losses from the viscera and heart muscle are less marked or insignificant Miller (1969) Hurley (1969) Huber and Gershoff (1970) Bergman (1970 a b) and others have discussed the hypotheses that the zinc concentrations in some of the viscera may be under homeostatic control and that the zinc in bone could function as a reserve for these zinc dependent tissues during periods of low zinc intake

In a previous article (Bergman *et al* 1974) we reported the patterns of the variation of zinc concentrations in the serum viscera heart incisors and 4 bone samples during growth in rats fed a conventional pellet diet The zinc concentrations in the bone samples are correlated to the mineral (ash) content in each and the zinc concentrations in the ash are correlated to the zinc concentration in serum In a com

parison of the turnover of ^{65}Zn and ^{85}Sr in rats (Wing 1974), one of us has demonstrated that much of the zinc in bones and teeth is accreted, or moved from an exchangeable pool to a fraction which is relatively unavailable for exchange The purpose of the present investigation was to compare the turnover of ^{65}Zn in samples from rats fed a zinc deficient diet with and without a zinc supplement in order to further examine the above hypotheses

Materials and Methods

Forty weanling female albino rats of the Sprague Dawley strain were used in the investigation The rats were randomly assigned to one of two groups of 20 rats each and these groups further subdivided into groups of 4 to be housed in the same cage The cages were constructed of acrylic resin From 3 weeks of age until the last group was killed both groups were fed a commercially available zinc deficient diet (Nutritional Biochemicals Cleveland Ohio Forbes and Yohe 1960) and 3x distilled water The two groups were paired fed that is an equal weight of food minus spill was given daily to pairs of cages one cage from each group Water was available *ad libitum* One of the groups 5 cages received a daily oral zinc supplement of 0 2 mg zinc as the sulfate in water The rats in both groups received an oral mineral supplement twice weekly (Millar *et al* 1958)

At 3 weeks of age eight of the rats 4 from each group and at 9 weeks of age the remaining 32 rats were injected i p with ^{65}Zn The ^{65}Zn as zinc chloride in 0 1 N HCl had a specific activity of 473 mCi ^{65}Zn/mg zinc (the Radiochemical Centre Amersham England) The injection solution was prepared by diluting the stock solution with physiologic saline to final concentrations of 80 μCi ^{65}Zn/ml injection solution for the first 8 rats and 40 μCi/ml for the remaining 32 rats The injected doses were 0 4 and 0 2 μCi per gr b wt respectively

Of the 8 rats injected at 3 weeks of age 2 from each group were killed at 4 weeks and the remaining 2 from each group killed at six weeks of age one and three weeks respectively after the start of the experiment and the ^{65}Zn injections Of the remaining 32 rats injected at 9 weeks of age after 6 weeks on the zinc deficient diet 4 rats from each group 1 from each cage were killed at 1 3 7 and 14 days post injection The animals were lightly anesthetized with ether and killed by decapitation The sampling techniques and preparation of samples for the determinations have been described earlier (Bergman 1970 c Bergman *et al* 1974) Samples of serum kidney pancreas spleen liver (median lobe) heart incisors (pulpless crowns) mandibular condyle mandibular bone (ramus and part of the corpus) tibia epiphysis and tibia diaphysis (bone marrow removed) were taken The tissue samples were removed using polyethylene or polyethylene covered instruments to avoid contamination by contact with metal Each sample was placed in a weighed Pyrex tube and weighed immediately Thereafter the samples were dried in an oven for 4 to 6 h at 100 C and then overnight at 150 C The samples were transferred to a muffle oven and the temperature was slowly raised and then maintained at 550 C for a second night The ash weights of the specimens were recorded after cooling to room temperature and the ash dissolved in 0 5 ml 10 5 % HCl overnight All samples were then diluted with 2 0 ml 3x distilled water (Bergman *et al* 1974)

The ^{65}Zn activity in the samples was measured in a 7 6×7 6 cm NaI (Tl) well scintillation detector with a single channel analyzer (AE 3707 Atomenergi Studsvik Sweden) calibrated to operate at the 1 116 MeV y peak of ^{65}Zn Known amounts of the injection solution were diluted to the same volume 2 5 ml to give comparable counting geometry and were counted on each occasion The backgrund counting rate was also measured on each occasion and subtracted from the counting rates for the tissue ash and injection solution samples

After the scintillation measurements the samples were further diluted with 2 % HCl as needed and the zinc concentration in each determined by atomic absorption spectrophotometry The determinations were made with a zinc lamp and a Unicam SP 90 spectrophotometer at 213 9 nm Reference samples of zinc in 2 2 % HCl were used The absorption for each of the mineralized tissue samples was corrected for the absorption due to the high calcium concentration by subtracting a further absorption measurement at 210 nm (Bergman *et al* 1974)

The relative activity R A of ^{65}Zn in a sample was calculated from the corrected sample and injection solution counting rates

$$R\,A = \frac{\text{cpm/g fresh weight of the sample}}{\text{cpm injected/g b wt}}$$

In the presentation of the data in the figures these values are multiplied by 100 to obtain the R A in percent The specific activity S A was calculated by dividing the R.A by the zinc concentration (mg Zn/g fresh weight) in each sample

The calculations of the RA and SA and their means and standard deviations as well as the analyses of variance were performed on an electronic computer (Control Data 3200 Umeå Datacentral) or a programmable calculator (Canon Canola 161P) using standard programs In the presentation of the results of statistical tests the following levels of significance are used

	$p \leqslant 0.001$	highly significant***
$0.001 <$	$p \leqslant 0.01$	significant**
$0.01 <$	$p \leqslant 0.05$	nearly significant*
$0.05 <$	p	not significant.

where p is the probability of incorrectly rejecting the null hypothesis

Results

The body weights in the 9 to 11 week old rats increase with increasing age in both groups (Table I) The analyses of variance are significant for the zinc supplement groups and nearly significant for the no supplement group The rats given the zinc supplement are slightly heavier The differences between the mean body weights of the 2 groups at the time of injection and between those of the 2 individual groups at each sacrifice time are not significant

In Table II are presented the means and standard deviations of the fresh weight zinc concentrations in the 11 samples from the 2 groups of 16 rats which were fed the zinc-deficient diet with and without a zinc supplement from 3 weeks of age They were injected with ^{65}Zn at 9 weeks of age and the samples were taken at 1 3 7 or 14 days post injection The coefficients of variation ($100 \cdot SD/\bar{x}$) in the serum viscera and heart in both groups average 9 % and are highest in pancreas and lowest in spleen and heart The coefficients of variation for the fresh weight zinc concentrations in the incisors and bone samples average 7 % in the zinc supplement group and 12 % in the no supplement group and are highest in mandibular condyle These concentrations are relatively stable between 9 and 11 weeks of age and the analyses of variance are significant only for the zinc concentrations in mandibular bone and tibia epiphysis in the zinc supplement group and for that in heart in the no supplement group

TABLE I The body weights of the 2 groups of rats fed a zinc-deficient diet from 3 weeks of age The weights to the left (0 days) are for the entire groups (n = 16) at the time of injection with ^{65}Zn at 9 weeks of age and the remaining 4 columns give the weights of the subgroups at sacrifice (n = 4) The results of t tests of the differences in weight between the groups appear below and the results of the analysis of variance on the weights at sacrifice within each group are given under F

	Days post injection 0		1		3		7		14		F
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
Zinc supplement	11[illegible] 8	9	119 9	13	1?0 0	13 1	130 1	11 1	131 5	[illegible]4	4 99 *
No supplement	110 7	9	11[illegible] 3	13 8	119 1	[illegible]0	118	10 4	143 0	1[illegible]	[illegible]93
t	1 [illegible]31		0 477		0 121		1 5?5		1 531		

TABLE II The fresh weight zinc concentrations in ppm (µg/g) in the eleven samples from 2 groups of 16 rats each fed a zinc deficient diet with and without a zinc supplement from 3 weeks of age and sacrificed in groups of 4 at 4 different times after injection with ⁶⁵Zn at 9 weeks of age The ash weight/fresh weight ratios and the ash weight zinc concentrations in the mineralized tissue samples are also given The means are those for all 16 rats in each group and the standard deviations are for the individual sacrifice groups (n = 4 within) and the entire groups (total) obtained in the analysis of variance on the 4 ⟵ sacri fice groups the results of which are given under F with th levels of significance The re sults of the t test on the difference between the 2 group means are also presented under t

	Zn suppl ment				No supplement				
	x̄	S D within	S D total	F	x̄	S D within	S D total	F	t
Zn ppm fresh weight									
serum	1 09	0 13	0 12	0 10	0 99	0 13	0 15	2 50	2 07*
kidney	26 0	1 4	1 4	0 79	21 5	0 8	0 9	2 70	11 17**
pancreas	18 5	2 5	2 5	1 29	16 4	3 4	3 2	0 46	2 13*
spleen	22 2	0 5	0 6	2 62	22 2	0 5	0 6	3 05	0 36
liver	26 9	3 5	3 5	0 94	23 4	2 7	3 4	4 16*	2 84 *
heart	18 1	0 7	0 7	0 26	17 3	0 3	0 5	9 35**	3 97**
incisors	88 0	5 5	6 8	3 68*	56 7	3 2	3 5	2 10	16 44 *
mand condyle	77 1	7 1	8 4	3 14	55 3	11 7	12 7	1 85	5 74 *
mand bone	95 3	6 3	9 4	7 25**	61 6	6 6	9 1	5 50*	10 79 *
tib epiphysis	76 0	4 6	7 8	10 22***	52 4	4 5	5 9	4 73*	9 61***
tib diaphysis	130 7	8 3	8 6	1 33	88 6	10 2	10 5	1 38	12 40 *
Ash weight fresh weight									
incisors	0 719	0 019	0 021	1 92	0 715	0 016	0 015	0 37	0 63
mand condyle	0 242	0 023	0 024	1 63	0 207	0 034	0 034	1 01	3 34 *
mand bone	0 460	0 038	0 040	1 43	0 492	0 047	0 054	2 48	2 83
tib epiphysis	0 190	0 012	0 014	2 96	0 191	0 009	0 015	11 39***	0 25
tib diaphysis	0 541	0 042	0 040	0.56	0 496	0 041	0 045	2 08	2.98
Zn ppm ash weight									
incisors	122 3	7 3	8 5	2 72	79 3	4 3	4 6	1 68	17 84 **
mand condyle	319 4	27 8	29 4	1 57	271 1	73 5	71 0	0 67	2.51
mand bone	207 3	13 6	15 6	2 58	146 8	16 6	17 4	1 53	10 35* *
tib epiphysis	399 9	15 5	21 0	5 17*	274 0	21 1	19 4	0 73	17 61
tib diaphysis	242 3	13 0	14 1	1 95	178 9	16 1	15 4	0 55	12 15 **

The zinc concentrations in the no supplement group are less *in all samples except* spleen The means for the entire groups (n = 16) were used in a t test (one tailed) of the differences between the zinc concentrations in the samples The dif ference is not significant in spleen the difference for serum pancreas and liver are nearly significant and those for the remaining samples are highly *significant* The least relative difference occurs in spleen and the greatest in incisor crowns The average relative difference for the mineralized tissues is 32 % of the concentration in the respective sample from the zinc supplement group *(range 27—36 %)*

The fresh weight zinc concentrations in the 11 samples from the 2 groups of two rats fed the zinc deficient diet with and without a zinc supplement for one week (4 weeks old) were higher than those from the respective group in *Table II* while those in the samples taken from rats after 3 weeks on *the diet (6 weeks old)* were equally as low as or lower than those in Table II

The means and standard deviations of the ash weight/fresh weight ratios and the ash weight zinc concentrations in the mineralized tissues from the 9 to 11 week old rats are also presented in Table II The coefficients of variation of the ash weight/fresh weight ratios average 7 % in the zinc supplement group and 9 % in the no supplement group and are highest in mandibular condyle and lowest in incisors There is a slight tendency for the ratios to increase with increasing age between 9 and 11 weeks The analysis of variance is nearly significant only for the tibia epiphysis from the no supplement group The ratios are lower in the samples from the no supplement group except in the tibia epiphysis The differences between the means for the two groups were tested with a t test and the differences are significant in mandibular bone mandibular condyle and tibia diaphysis The coefficients of variation of the ash weight zinc concentrations in these samples average 6 % in the zinc supplement group and 12 % in the no supplement group and are highest in the mandibular condyle These concentrations show no trend with increasing age and the analysis of variance is significant only for that in tibia epiphysis in the no supplement group The ash weight zinc concentrations are lower in the samples from the no supplement group The t tests for the differences between the means for the groups are highly significant for all but the mandibular condyle for which the t value is significant The relative differences in the concentrations average 27 % of the concentration in the respective sample from the zinc supplement group (range 11—35 %)

The ash weight/fresh weight ratios in mineralized tissue samples from the 4 and 6 week old rats are lower than those for the samples from the 9 to 11 week old rats in both groups Those from the 4 week old rats are generally lower than those from the 6 week old rats The ash weight zinc concentrations are higher in the mineralized tissue samples from the 4 week old group than in those from the 9 to 11 week old group These concentrations are lower in the 6 week old rats and in most samples are as low as those in the 9 to 11 week old rats

The mean ^{65}Zn R A in percent in these same samples from the 9 to 11 week old rats are presented in Fig 1 The coefficients of variation for the R A in all eleven samples from both groups at all 4 times post injection average 11 % (range 1—45 %) and are greatest in incisors and mandibular condyle and least in kidney and heart The difference between the mean R A of the 2 groups for each sample and sacrifice time were tested with a t test The greater number of significant differences occur at 7 and 14 days post injection and significant differences are especially frequent in the serum viscera and heart The R A in the serum of the no supplement group is not significantly different from the R A in the serum of the zinc supplement group at 1 and 3 days post injection but at 7 and 14 days it is highly significantly greater The R A in the viscera of the no supplement group are higher and the R A in the mineralized tissues lower than the corresponding R A for the zinc supplement group The relative differences in the R A in the mineralized tissues average [illegible] % of the respective zinc supplement group R A at 1 day (range 15—

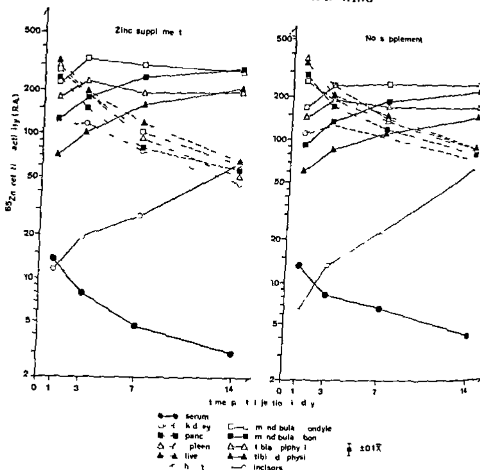


Fig 1 The ^{65}Zn relative activities R A in the serum and selected tissues from rats fed a zinc deficient diet from 3 weeks of age and injected with ^{65}Zn at 9 weeks of age One group received a daily zinc supplement (left) the other did not *N B* log scale for R A *Each point* represents the mean of the R A in samples from 4 rats The bar represents a coefficient of variation (100 S D /x) of 10 %

45 %) and this difference decreases slightly in mandibular condyle and markedly in incisors and increases in tibia diaphysis with time

The ^{65}Zn S A was calculated for each sample by dividing the R A by the zinc concentration in mg/g fresh weight of the sample The mean S A are presented in Fig 2 The coefficients of variation are generally slightly higher than those for the corresponding R A The average coefficient is 12 % (range 1—59 %) with the highest in mandibular condyle and the lowest in kidney The difference between the mean S A of the two groups for each sample and sacrifice time were tested with a *t* test The serum S A in the two groups are practically equal at 1 day post injection and those in the serum of the no supplement group are highly significantly greater at 3 7 and 14 days The S A in most of the viscera follow the respective group serum S A and are nearly equal to it The differences between the group

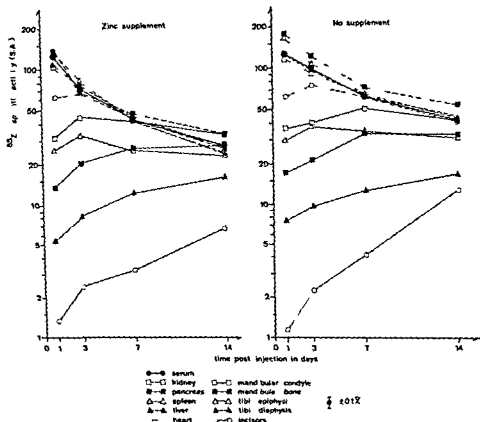


Fig 2 The ^{65}Zn specific activities S A (R A /(mg Zn/g)) in the serum and selected tissues from rats fed a zinc-deficient diet from 3 weeks of age and injected with ^{65}Zn at 9 weeks of age One group received a daily zinc supplement (left) the other did not. N B log scale for S A Each point represents the mean of the S.A. in samples from 4 rats The bar represents a coefficient of variation (100 SD /x) of 10 %

mean S A are with two exceptions highly significant for the viscera and heart at 3 7 and 14 days At 1 day post injection the S A in the pancreas and spleen from the no supplement group are higher than the serum S A and the S A in pancreas maintains a level approximately 25 % higher than the serum S A throughout the experimental period At 1 day the S A in the hearts from the two groups are nearly equal at 50 % of the serum S A The S A in the heart of the zinc supplement group achieves the level of the serum S A by 3 days while that in the no supplement group takes longer time to do so Both then follow the respective serum S A The S A in the bone samples from the no supplement group at 1 day are somewhat higher than the corresponding samples from the zinc supplement group The difference in the S A in the tibia diaphysis slowly diminishes with time The S.A in incisors at 1 day are nearly equal and the S A at 7 days is larger in the no supplement group and still larger by 14 days

One of the 6 week old rats in the no supplement group has been eliminated from the R.A and S A data as the R.A in the samples from this rat were only one tenth of those in its mate The R.A in the samples from the 4 week old rats (1 week post injection) given the zinc supplement are in all eleven samples higher than those in the corresponding samples from the no supplement group With the exception of the R.A in tibia epiphysis and diaphysis, the relationship is exactly the opposite in the 6 week old rats (3 weeks post injection) The R A at 3 weeks post injection in the samples from the zinc supplement group are considerably lower than the R.A at 1 week except in teeth in which they are higher, and tibia diaphysis in which they are only slightly lower The R A at 3 weeks post injection in the samples from the no supplement group are only slightly lower than the 1 week levels in most samples and are higher in incisors mandibular bone and tibia diaphysis The S A follow nearly the same pattern being higher in all 11 samples from the zinc supplement group at 4 weeks of age and lower at 6 weeks of age The S A in all samples within each group are very much alike at 3 weeks post injection (6 weeks of age)

Discussion

The methods and method errors have been discussed at length in previous reports (Bergman 1970 a c Bergman *et al* 1974 Wing 1974) Ashing the serum samples at 550° C results in a loss of 15—20 % of the zinc to the Pyrex glass tubes (Bergman *et al* 1974) which in turn results in an overestimation of the ^{65}Zn specific activities S A. in serum by approximately 20 % These errors can be corrected by multiplying the serum zinc concentrations by 1 2 and the S A. by 0 83 Except for this error the errors in the methods appear to be both random and very small The ^{65}Zn data from one rat injected at 3 weeks of age were eliminated due to an injection error

The growth of the rats fed the zinc deficient diet is well below normal though growth is not completely inhibited The growth of the rats pair fed the same diet and given a daily supplement of 200 μg zinc is not significantly greater perhaps due to restricted food intake These results differ from those of an earlier investigation in our laboratory using the same experimental design but a diet containing only 1 3 μg zinc/g (Bergman 1970 a) In the earlier investigation growth was negligible in the no supplement group and the rats which received the supplement had significantly higher body weights Thus the diet used in the present study is not zinc deficient in the extreme

The zinc concentrations in the samples from the no supplement group are generally lower than those in the zinc supplement group the differences being greatest for the bone samples These results are in agreement with those of earlier studies (Macapinlac *et al* 1966 Prasad *et al* 1967 Swenerton and Hurley 1968 Huber and Gershoff 1970 Bergman 1970 a) At 9 to 11 weeks of age, the fresh weight zinc concentrations in the bones of the rats in the zinc supplement group average 30 % less and those from the no supplement group 50 % less (ranges 10—40 %

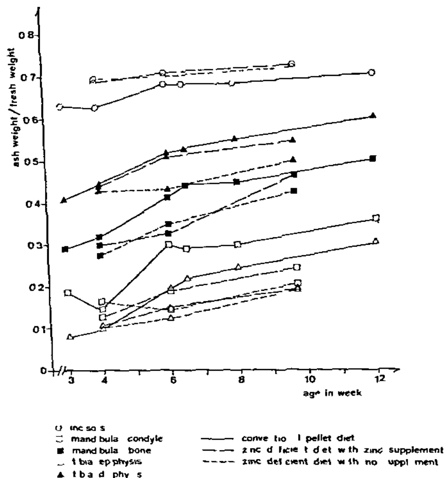


Fig 3 The mean ratios of the ash weight to fresh tissue weight in 5 mineralized tissue samples from rats fed a conventional pellet diet (Bergman *et al* 1974 Wing 1974) and from rats fed a zinc deficient diet with or without a daily zinc supplement (from 3 weeks of age (present study)

and 45—53 %) than those in the bones of rats of approximately the same a conventional pellet diet containing 70—75 μg zinc/g (Bergman *et al* 1 net mineralization in the bones differs among these three groups (Fig 3) increases in mineral content between 3 and 6 weeks of age in the ventional diet group being absent in all but the tibia diaphyses from ment group While these differences cannot be directly ascribed to zinc intake they are most marked in the no supplement group The concentrations in the bones of the two groups the zinc-d during the first few weeks after weaning assume a

decreases are more rapid and more marked than those in the conventional diet group At 9 to 11 weeks of age, the ash weight zinc concentrations in the zinc supplement group average 16 % less and in the no supplement group 36 % less than those in the bones of the conventional diet group (ranges 10—27 % and 24—46 %) The stabilization of these concentrations in all three groups may represent new steady states in the turnover of zinc in bone with respect to the different zinc intakes in the period after weaning As the differences in the ash weight zinc concentrations in the bones are greater than the respective differences in the serum concentrations it may be that not all the zinc in serum is available to the bone The high zinc concentration in the incisors of the zinc supplement group may have been caused by contamination during administration of the supplement with a pipette The high mineral contents in the incisors of both groups may indicate a change in the eruption pattern or other factors which could affect the relative proportions of enamel and dentin

The general patterns of the turnover of ^{65}Zn in the eleven samples from the groups fed the zinc deficient diet with and without the zinc supplement are quite similar The ^{65}Zn specific activities S.A in the viscera follow the serum S A very closely The S A in the pancreas of the no supplement group maintains a level 50 % higher than the serum S A (corrected value) This large a difference in an organ in which the S A so rapidly achieves the level of the serum S A should indicate that the rapidly exchanging zinc in the pancreas is greatly reduced leaving that fraction which is accumulated and relatively unavailable for exchange to dominate the ^{65}Zn turnover pattern It would appear likely that the freely exchangeable zinc in the pancreas is also the zinc which is loosely bound and varies with the serum zinc concentration (Bergman *et al* 1974) The turnover of ^{65}Zn is most rapid in the acinar cells and much slower in the cells of the islets of Langerhans (McIsaac 1955) Thus it could be that the loosely bound freely exchangeable zinc is that which is being excreted by the pancreas while the fraction which is accumulated for a time may represent zinc which is important to the synthesis, storage and release of insulin (Falkmer 1971)

At 1 day post injection the S.A in both the serum and the hearts of the two groups are nearly equal indicating that no change occurs in the turnover rate for the heart That the S A in heart takes a longer time to reach the level of the serum S A in the no supplement group is most likely due to the higher serum S A in that group at times later than 1 day The turnover of ^{65}Zn in the viscera other than pancreas relative to the turnover in serum appears to be unaffected by the difference in zinc intake The patterns represent a rapid passive equilibration in these organs the rate of exchange in the spleen being slightly lower than those in the kidney and liver

It would appear likely that the courses of the serum S A prior to 1 day post injection in the groups fed the zinc deficient diet are quite similar and approach the course of the serum S A in rats fed a conventional diet (Wing 1974) (Fig 4) This might be expected as much of the rapidly exchanging zinc in the body is not

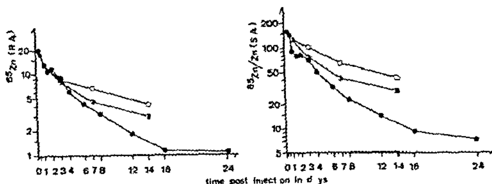


Fig 4 The mean ^{65}Zn relative activities R A (left) and specific activities S A in the serum from rats fed a conventional pellet diet and injected with ^{65}Zn at 6 weeks of age (filled circles Wing 1974) and from rats fed a zinc-deficient diet with (half filled circles) or without (open circles) a zinc supplement from 3 weeks of age and injected with ^{65}Zn at 9 weeks of age (the latter data are the same as those in Fig 1 and 2 respectively) N B log scales for R A and S A

disturbed by decreasing the zinc intake Using graphically obtained time integrals of the serum S A made on the assumption that the early (0—0 5 day) courses in the groups fed the zinc-deficient diet are the same as that for the conventional diet group the amount of zinc available for exchange (E) and that accreted or made unavailable for exchange per day (A) were estimated for each bone sample using the retention data from 1 and 3 days post injection and solving the Bauer Carlsson Lindquist equation (1955) as two simultaneous equations

$$R_t = E \cdot S_t + A \int^t S_t \, d_t$$

where R_t is the ^{65}Zn R A in the bone and S_t is the serum S A at time t The results of this analysis are presented in Table III where they are compared to the values obtained from 6 week old rats fed a conventional diet (Wing 1974) All values are based on 1 gram fresh weight of bone The validity of such analysis in estimating the turnover of zinc in bone is discussed in the report of a study in which the retention of ^{65}Zn in bone is compared with that of ^{85}Sr (Wing 1974)

The E and A values for each bone sample in all three groups are generally very much alike when expressed as percent of the fresh weight zinc concentration in the respective sample The A values for the zinc supplement group average 33 % lower and those for the no supplement group 56 % lower than the respective A values for the conventional diet group (ranges 30—36 % and 50—61 %) These differences are larger than the differences in the ash weight zinc concentrations As the mineral content in the bones of the rats fed the zinc deficient diet is lower than that in the rats fed the conventional diet and as Hurley *et al* (1969) have demonstrated decreased turnover of calcium in the bones of zinc deficient rats it is likely that these decreased accretion rates represent decreases in the accretion of bone as well

TABLE III The values for the exchangeable pool size E and the accretion rate A in the bone samples from rats fed a zinc deficient diet with and without a zinc suppl ment as calculated using the data on ^{65}Zn retention at 1 and 3 days post injection in the Bauer Carlsson Lindquist equation The values calculated using the data from an earlier study of rats fed a conventional diet are presented for comparison All values are bas d on 1 gram fresh weight of bone The E and A values have been divided by the respective fresh weight zinc concentrations to give the values in percent

	E mg Zn	A mg Zn day^{-1}	E % Zn	A % Zn day^{-1}
Zinc supplement				
mand condyle	0088	0079	11 4	10 2
mand bone	0053	0040	5 6	4 [illegible]
tib epiphysis	0086	0043	11 3	[illegible] 4
tib diaphysis	0079	0023	2 2	1 8
No supplement				
mand condyle	0082	0043	14 8	1[illegible] 8
mand bone	0045	0024	7 3	3 9
tib epiphysis	0078	0030	14 9	5 8
tib diaphysis	0030	0015	3 4	1 7
Conventional diet[1]				
mand condyle	0265	0118	22 1	9 8
mand bone	0103	0056	7 8	4 2
tib epiphysis	0108	0064	12 6	7 5
tib diaphysis	00[illegible]8	0042	4 2	2 6

data from Wing 1974

Using the calculated E and A values and the time integral of the serum S A through 7 days post injection the predicted retention at 7 days for mandibular bone and tibia diaphysis in both groups fed the zinc deficient diet are very close to the observed R A indicating that slowly exchanging zinc compartments (Wing 1974) are very much reduced due to the lower zinc intakes This is taken to be evidence that the slowly exchanging fractions are separate from the accreted zinc In this connection it is interesting to compare the three serum S A curves on log log scales (Fig 5) The curve for the group fed the conventional diet is seen to turn downward at 3 days post injection and follow a course with a slope which would have the serum S A approximately proportional to the inverse of time Marshall (1969) uses this course for other bone seeking radionuclides as part of the evidence for the existence in bone of slowly exchanging compartments which imitate the accreted fraction of activity retention at earlier times post injection There is no apparent tendency for the serum S.A curves for the two groups fed the zinc deficient diet to follow such a course This may further indicate that such slowly exchanging compartments for zinc are insignificantly small in the bones of zinc-deficient rats

Our observations agree well with those of Miller (1969) Cotzias *et al* (1962) and Cotzias and Papavasiliou (1964) on ^{65}Zn turnover in ruminants and mice respectively However while these authors interpret their data as indicating that the viscera develop a greater affinity for zinc as a result of zinc deficiency and that the turnover of zinc is under homeostatic control at the sites of absorption and ex

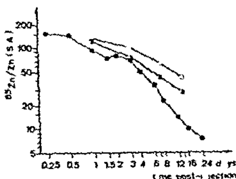


Fig 5 The mean ^{65}Zn specific activities S.A in the serum from rats fed a conventional pellet diet (filled circles) and a zinc deficient diet with (half filled circles) and without (open circles) a daily zinc supplement The data are the same as those in Fig 4—right. N.B log scales for both S.A. and time post injection.

cretion our interpretation is that the turnover of zinc is a predominantly passive process and very much similar in the rats fed the conventional and zinc-deficient diets The increased ^{65}Zn S.A in the serum, heart and viscera in the groups fed the zinc-deficient diet appear to be the direct result of low zinc intake and resultant decreased excretion limiting the amount of zinc available to exchange with the ^{65}Zn The maintenance of the zinc concentrations in several viscera and heart despite low zinc intake may be an expression of the strength of the binding of zinc to RNA DNA and enzymes in these tissues (Prasad *et al* 1967) The turnover of zinc in bone appears to be proportional to the rate of bone accretion and the amount of zinc available via the serum Further as zinc is important both to the synthesis of RNA DNA and protein (Prasad *et al* 1971) and to the mineralization of bone (Hurley *et al* 1969) the availability of zinc may indirectly regulate the zinc concentrations by limiting growth and accretion which would have diluted the zinc concentrations

As much of the body s zinc is bound in the bone it is not readily available as a reserve during periods of low intake or increased demand such as in healing but is released from bone only as fast as the bone itself is resorbed A small exchangeable fraction of the zinc in bones and teeth is perhaps available as a more immediate reserve Thus a tissue in need of zinc must be supplied with exogenous zinc for normal growth function or repair

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References

Bauer G C H A Carlsson and B Lindquist Evaluation of accretion resorption and exchange reactions in the skeleton *Kungl Fysiogr Sallskap Lund Fo handl* 1955 25 3—18

Bergman B The zinc concentration in hard and soft tissues of the rat The influence of zinc deficient feeding *Acta odont scand* 1970a 28 425—440

Bergman B The distribution and concentration of zinc and the effect of zinc deficiency in the mammalian body *Odont Revy* 1970b 21 Suppl 20

BERGMAN, B., Concentration of zinc in some hard and soft tissues of rat determined by neutron activation analysis. *Acta radiol. Ther. Phys. Biol.* 1970 c. *9* 420—432.
BERGMAN, B., R. SJÖSTRÖM and K. R. WING, The variation with age of tissue zinc concentrations in albino rats determined by atomic absorption spectrophotometry. *Acta physiol. scand.* 1974 *92* 440—450
COTZIAS, G. C. and P. S. PAPAVASILIOU, Specificity of zinc pathway through the body: homeostatic considerations. *Amer. J. Physiol.* 1964 *206* 787—792
COTZIAS, G. C., D. C. BORG and B. SELLECK, Specificity of zinc pathway through the body: turnover of Zn^{65} in the mouse. *Amer. J. Physiol.* 1962 *202* 359—363
FALKMER, S., Sulfhydryl compounds and heavy metals in islet morphology and metabolism. *Proc. VII Congr. Internat. Diab. Fed. Buenos Aires Aug. 1970.* Exc. Med. Internat. Congr. Ser. 1971 *231* 219—225
FORBES, R. M. and M. YOHE, Zinc requirement and balance studies with the rat. *J. Nutr.* 1960 *70* 53—57
HUBER, A. M. and S. N. GERSHOFF, Effects of dietary zinc and calcium on the retention and distribution of zinc in rats fed semipurified diets. *J. Nutr.* 1970 *100* 949—95
HURLEY, L. S., Zinc deficiency in the developing rat. *Amer. J. clin. Nutr.* 1969 *22* 133—1339
HURLEY, L. S., J. GOWAN and G. MILHAUD, Calcium metabolism in manganese-deficient and zinc-deficient rats. *Proc. Soc. exp. Biol. (N.Y.)* 1969 *130* 856—860
MACAPINLAC, M. P., W. N. PEARSON and W. J. DARBY, Some characteristics of zinc deficiency in the albino rat. In *Zinc Metabolism* (ed. Prasad, A.S.) Charles C. Thomas Publ., Springfield Ill. 1966 pp. 142—168
MARSHALL, J. H., Measurements and models of skeletal metabolism. In *Mineral Metabolism* Vol. III (ed. Comar, C. L. and F. Bronner) Academic Press, New York 1969 pp. 1—122
McISAAC, R. J., The distribution of zinc-65 in the rat pancreas. *Endocrinology* 1955 *5*[illegible] 571—579
MILLAR, M. J., M. I. FISHER, P. V. ELCOATE and C. A. MAWSON, The effects of dietary zinc deficiency on the reproductive system of male rats. *Canad. J. Biochem. Physiol.* 1958 *36* 557—569
MILLER, W. J., Absorption, tissue distribution, endogenous excretion, and homeostatic control of zinc in ruminants. *Amer. J. clin. Nutr.* 1969 *22* 1323—1331
PRASAD, A. S., D. OBERLEAS, P. WOLF and J. P. HOROWITZ, Studies on zinc deficiency: Changes in trace elements and enzyme activities in tissues of zinc-deficient rats. *J. clin. Invest.* 1967 *46* 549—557
PRASAD, A. S., D. OBERLEAS, E. R. MILLER and R. W. LUECKE, Biochemical effects of zinc deficiency: Changes in activities of zinc-dependent enzymes and ribonucleic and deoxyribonucleic acid content of tissues. *J. Lab. clin. Med.* 1971 *77* 144—152
SWENERTON, H. and L. S. HURLEY, Severe zinc deficiency in male and female rats. *J. Nutr.* 1968 *95* 8—18
WING, K. R., A comparison of the turnover of ^{65}Zn and ^{85}Sr in growing rats. *Acta radiol. Ther. Phys. Biol.* 1974 In press.

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From the Department of Clinical Physiology University of Umea Sweden

Relationship between Perivesical and Intravesical Urinary Bladder Pressures and Intragastric Pressure

By

PER BJERLE

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Abstract

BJERLE P *Relationship between perivesical and intravesical urinary bladder pressures and intragastric pressure* Acta physiol. scand 1974 92 465—473

The intragastric pressure and the perivesical and intravesical urinary bladder pressures were measured in the supine and sitting positions immediately after micturition in 9 healthy men. The intragastric pressure was measured with an air filled rubber balloon the perivesical pressure with an open liquid filled catheter inserted above the pubic bone into the space of Rezius and the intravesical pressure of the almost empty bladder with a catheter inserted suprapubically There was no difference between the intragastric pressure level and the peri and intravesical pressure levels in the supine position, but in the sitting position the peri and intravesical pressure levels were higher than the intragastric pressure levels due to the pressure effect of the viscera. There was no difference between perivesical and intravesical pressure levels in either the supine or sitting position Rapid pressure change produced by straining or coughing produced a smaller pressure increase peri and intravesically than intragastrically due to delayed pressure equilibration because of inhomogeneity of the abdominal contents

The pressure in the urinary bladder undergoes periodic variations as the result of the bladder s two functional states—that of a reservoir and that of an excretory organ While urine collects the pressure in the bladder is low during the phase of emptying the pressure is high due to contraction of the detrusor Since the bladder is located just below the abdominal cavity proper and is separated from the latter only by the peritoneum pressure changes in the abdominal cavity will obviously affect the intravesical pressure Posture and the tone of the musculature of the abdominal wall the musculature of the perineum and the diaphragm are factors that determine the intraabdominal pressure and thereby also affect the pressure in the bladder

Registration of the intravesical pressure can supply information on the functional state of the bladder wall provided that the intraabdominal pressure is known Recorded alone without simultaneous measurement of the intraabdominal pressure the intravesical pressure has been related to the bladder volume in a number of investigations (Dubois 1876 Mosso and Pellacani 1882 Denny Brown and Robert

son 1933, Juul Jensen 1962). There have also been numerous attempts to measur the intraabdominal pressure at the same time as the intravesical pressure. Pressure have been recorded by means of a balloon in the stomach (Backman *et al* 1966, von Garrelts 1957, Sundblad 1971), via a balloon (Gleason and Lattimer 1964, Susset *et al* 1965, Wise *et al* 1968, Gierup 1970 a, Gierup *et al* 1970), cathete (Claridge and Shuttleworth 1964, Claridge 1966, Frimodt Moeller 1972) or radio transmitter (Warrell *et al* 1963) in the rectum or by means of an extraabdomina tocodynamometer (Smith *et al* 1966, Rowan *et al* 1972). Subtraction of the intra gastric or intrarectal pressure from the intravesical pressure, in order to obtain the true so-called detrusor pressure, has been done mechanically (Gleason and Lattimer 1964) or by means of subtraction circuits in the recording apparatus (Gleason and Lattimer 1964, Backman *et al* 1966, Sundblad 1971, Frimodt Moeller 1972).

The methods for measuring the contribution of the intraabdominal pressure to the intravesical pressure thus far described in the literature suffer from certain in adequacies. Recording of the intragastric pressure fails to take into account the hydrostatic pressure component due to the abdominal viscera (Sundblad 1971). Further, the intragastric pressure is dependent on the degree of filling of the stomach (Koester and MacLen 1970). It has been pointed out that the rectal pressure does not permit valid quantitation (Gierup 1970 b). Muscular activity in the wall of the rectum can lead to recording of falsely high pressures (Denny Brown and Robert son 1933, Rushmer 1946). Extraabdominal pressure recording gives only relative pressures (Smith *et al* 1966).

In order to determine the transmural bladder pressure it would be desirable to be able to record the pressure immediately outside the bladder wall. To this end a modified method of perivesical pressure measurement has been developed and the values obtained are compared with other measurements of extravesical pressure and with intravesical pressure.

Material

The material consisted of nine male volunteers, 30—58 years of age, mean age 39 years. All were in good health and had no symptoms or signs suggestive of pathology of the urinary tract. None had had disturbances of micturition in the form of weak urine stream, difficulty in initiating micturition, pain at urination or dribbling. They had no history of prostatitis or peritonitis. None had undergone abdominal surgery. Prior to the investigation the subjects underwent a routine physical examination including rectal palpation of the prostate. The urine sediment was examined microscopically and urine culture showed the urine to be free of bacteria. The experimental subjects were told how the trial was to be conducted and gave their full consent.

Methods

Procedure

Intragastric pressure. When the intragastric pressure is to be registered as a measure of the pressure in the upper part of the abdominal cavity, the recording balloon should be placed in the fundus of the stomach. To assure good definition of the site of pressure measurement regardless of the size of the stomach, a balloon with a fairly large volume should be used. It w

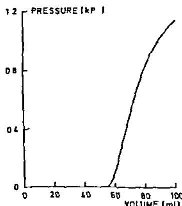


Fig 1 Relationship between pressure and volume during inflation of air in the latex balloon used for registering intragastric pressure

remain in the fundus which is usually filled with air and not slip down into the antrum where it may be more affected by hydrostatic pressure changes especially in the sitting position Neither can a relatively large balloon, once inflated slip back into the esophagus A balloon with a volume of about 50 ml was therefore constructed

Through a nostril a Teflon catheter fitted at the tip with a Latex balloon was advanced into the stomach The balloon was 8 cm long and 3 cm in diameter The catheter had several lateral perforations in the part which was within the balloon The balloon was filled with 40 ml of air It could be filled to 55 ml without exceeding a pressure of 0 1 kPa (Fig 1)

Intravesical pressure After the subject's bladder had been allowed to fill spontaneously with urine for 4—5 h and could be identified by percussion and palpation above the symphysis a puncture was performed

Suprapubic puncture carries a risk of leakage into the perivesical space, but is nonetheless preferable to urethral catheterization for recording of the intravesical pressure

The leakage which occasionally occurs dos not give rise to any major discomfort or permanent damage (Nystrom *et al* 1973) Urethral catheterization on the other hand is attended by considerable risk of infection (Kass and Sossen 1959 Baumann and Schmid 1967 Desautel 1969) and the presence of the catheter together with the swelling of the urethral mucosa to which it gives rise makes determination of urethral resistance unreliable if micturition analysis is to be performed (von Garrelts 1968)

With the subject supine and after sometimes shaving some of the pubic hair the lower part of the abdomen was carefully washed and covered with sterile drapes except for an area above the symphysis about the size of the palm of a hand Immediately cranial to the symphysis the skin and subcutis but not the bladder wall were anesthetized Using the Seldinger technique (Seldinger 1953) two Teflon catheters PE 160 *i e* with outer diameter 1 6 mm and inner diameter 1 0 mm with 4—5 lateral perforations in the distal 3 cm were inserted 7—8 cm into the bladder A little urine was aspirated through the catheters The points of insertion were 1—2 cm apart. One of the bladder catheters was attached to an infusion set containing NaCl solution for cystometric investigation which was performed in connection with this trial (Bjerle a and b)

Perivesical pressure Positioning of a perivesical catheter does not involve perforation of the bladder wall and is attended by minimal risk to the subject, provided that the catheter is not advanced toward the proximal urethra where the plexus vesicopudendalis can be damaged

One cm cranial to the suprapubic puncture sites a Seldinger needle was inserted in the direction toward the points where the catheters penetrated the bladder wall When the tip of the needle rested against the bladder wall a Teflon guide was inserted to the same depth whereupon the needle was removed leaving the guide in place The depth of the needle was noted and marked with tape on another catheter with a hole at the tip and two small lateral holes 2 mm from the tip This catheter was threaded over the guide until the tape rested against the skin whereupon the guide was withdrawn The catheter was filled with a sterile solution of 0 9 % NaCl

Pressure registration The three catheters from the stomach the space of Retzius and the urinary bladder were attached to the transducer manometer systems The subject was allowed to void Thereafter 0 5 ml NaCl solution was injected through the perivesical catheter to create

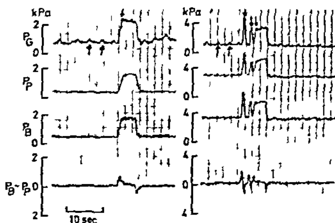


Fig 2 Example of pressure registration in subject G G in the supine and sitting positions P_G = intragastric P_I = perivesical P_I = intravesical and P_P—P_P = differential pressure ↑ denotes inspiration ↓ straining

a pool of liquid around the tip of the catheter and prevent the three holes being occluded by contact with the perivesical tissue To check that there were no tiny air bubbles in the system and that the transmission was perfect, the subject was asked to breathe deeply strain or cough If the resulting deflections were approximately equal in size and congruent in shape reproduction was considered to be satisfactory and the pressures were continuously recorded for a time with the subject either supine and then sitting or for 5 of the subjects vice versa. Between the 2 recordings the subject was instructed to void

The intragastric pressure was related to atmospheric pressure For the perivesical and intravesical pressures the 0-reference level was at the superior border of the symphysis in the sitting position and in the supine position at the level of skin puncture since the latter could be determined before the start of the experiment To the pressure recorded in the supine position was added the pressure of a column of water of the same height as the vertical distance from the point of puncture to the point where with the needle tip the anterior wall of the bladder had been estimated to be situated

In confirmation with the Systeme International d Unites pressure is given in Pascal (Swedish Standardization Board 1971) 1 kPa corresponds to 7 5 mm Hg

The registration apparatus consisted of mechano-electrical transducers with amplifiers and a direct writing ink oscillograph (EMT 34 EMT 31 and Mingograph 81 made by Siemens-Elema, Stockholm, Sweden) It also contained a subtraction unit by which the difference between two pressures could be obtained The volume displacement of the catheter transducer unit was 1 6 mm³/10 kPa. With a liquid filled system including the catheter the response to a sine wave pressure including the response of the subtraction unit was of correct amplitude and without resonance up to a frequency of 15 Hz. Before during and after pressure registration the system was calibrated with a hydrostatic standard The oscillograph deflections including the difference from the subtraction unit were proportional to the pressures applied

Statistical evaluation The mean and standard deviation were calculated for the pressures measured at the respective points in the supine and sitting positions The differences between paired values i.e the pressures in a given subject under the two different conditions of measurement were calculated and the means of the differences are given The probability that the differences were significant was investigated using Student's t test.

Results

Fig 2 shows an example of the intragastric perivesical and intravesical pressures recorded with the subject supine and in the sitting position The variations in pressure due to breathing and light straining are clearly seen The deflections were some

TABLE I Increase in intragastric (P_G) perivesical (P_P) and intravesical (P_B) pressures in the supine and sitting positions during light coughing or straining in seven healthy men

Subject	Pressure kPa							
	Supine				Sitting			
	Bladder volume	P_G	P_P in of P_G	P_B in of P_G	Bladder volume	P_G	P_P in of P_G	P_B in of P_G
G P	[illegible]20	3 67	96	86	300	2 33	84	73
R L	375	1 80	80	96	200	2 00	100	92
S.Å A	275	3 13	85	96	200	2 49	94	88
K A	220	5 53	94	97	425	1 40	79	79
P A L	75	2 10	85	86	25	2 24	87	95
K E B	125	1 60	88	88	100	4 00	99	93
G G	350	1 48	86	88	300	1 85	74	66
Mean			87 [illegible]**	91 0 *			88 1*	83 7**

* = 0 05 ≥ p > 0 01 ** = 0 01 ≥ p > 0 001 where p is the probability that the difference between intragastric pressure and peri-/intravesical pressure is caused by random factors

what less pronounced in the perivesical and intravesical recordings than in the intragastric recording Moreover a brief strain sometimes resulted in a biphasic deflection of the differential pressure between the intravesical and perivesical pressures while during a strain maintained for about 2 s or more the differential pressure returned to the initial value In seven subjects the deflection was first positive and then negative in two subjects this order was reversed Table I shows the part of the intragastric pressure increases that could be registered perivesically and intravesically in seven of the subjects during light coughing or straining in both supine and sitting positions during subsequent cystometry In both positions the perivesical and intravesical pressure increases were less than the intragastric increase With the exception of the initial biphasic deflection there was no difference between the perivesical and intravesical pressure increases during coughing either in *the supine or the sitting* position

The resting pressures at expiration measured in the empty or nearly empty bladder (≤ 50 ml) in the supine and sitting position are shown in Table II All pressure levels *show interindividual differences*

In the supine position the intragastric pressure level was 0 70 ± 0 24 kPa mean and S D the perivesical pressure level 0 82 ± 0 26 kPa and the intravesical pressure level 0 87 ± 0 20 kPa thus there was no true difference between the perivesical and *the intravesical pressures* The intragastric pressure level did not differ from the perivesical and intravesical pressure levels

In the sitting position the intragastric pressure level was 0 91 ± 0 21 kPa mean and S D the perivesical pressure level 2 55 ± 0 37 kPa and the intravesical pressure level 2 63 ± 0 38 kPa Thus in the sitting position the intragastric pressure was lower than the perivesical and intravesical pressure There was no difference between the perivesical and intravesical pressures in the sitting position

TABLE II Bladder volume needle depth intragastric (P_G) perivesical (P_P) and intravesical (P_B) of the pressures and the difference between the intravesical and perivesical pressure or symptoms of disturbed micturition

Subject	Age years	Bladder volume ml	Needle depth cm	Pressure level kPa Supine			Sitting
				P_{Gsu}	P_{Psu}	P_{Bsu}	P_{Gsit}
O B	42	0	7	0 27	0 81	0 81	0 98
G P	38	0	8	0 64	0 85	0 85	0 76
R L	31	50	6	0 60	0 32	0 72	0 55
S Å A	43	0	8	1 06	0 96	0 98	1 01
K A	58	0	9	0 72	1 22	1 22	1 18
H O	36	0	7	0 56	0 57	0 61	0 80
P A I	30	0	7	0 78	1 01	1 11	1 20
K-E B	33	0	5	1 02	0 75	0 85	0 87
G G	42	0	7	0 65	0 92	0 68	0 84
Mean	39			0 70	0 82	0 87	0 91
Standard dev				0 24	0 26	0 20	0 21
p							

n s = $p > 0.05$ *** = $p < 0.001$ where p is the probability that the difference is caused by random factors

The perivesical and intravesical but not the intragastric pressure levels were higher in the sitting position than with the subject supine With a change in body posture from the horizontal to the sitting position the intravesical pressure rise did not differ from the perivesical pressure rise

Discussion

Since all the subjects were in good health and had no symptoms or signs suggestive of abdominal or genitourinary tract pahology, the interindividual differences in the respective pressures reported probably reflect normal variation

According to Guyton *et al* the total tissue pressure is the sum of solid tissue pressure and interstitial fluid pressure By measuring the pressure with an open microcatheter or a glass capillary filled with liquid injected in excess so that there is a little collection of liquid around the tip of the catheter or capillary the total tissue pressure at the point of measurement is obtained (Guyton *et al* 1971) Since the tip of the catheter was positioned at the bladder wall the perivesical pressure was measured at a point anterior to the bladder Were this pressure an expression of tissue properties alone it should have been constant for different body postures However it was higher in the sitting than in the supine position which means that a hydrostatic component was added to the existing tissue pressure The pressure in the abdominal cavity was thus transmitted to the extravesical space but the h

pressures in the supine position (su) and in the sitting position (si) the differences between some increases with a change from the horizontal to the sitting position in nine men with no signs

P_{PSI}	P_{BSI}	$P_{GSU}-P_{PSU}$	$P_{GSI}-P_{GSU}$	$P_{PSI}-P_{PSU}$	$P_{BS}-P_{BSU}$	$P_{BSI}-P_{BSU}-(P_{PSI}-P_{PSU})$
2 29	2 37	−0 54	0 71	1 48	1 56	0 08
2 54	2 54	−0 21	0 12	1 69	1 69	0 00
2 14	2 22	0 28	−0 05	1 82	1 50	−0 32
2 23	2 39	0 10	−0 05	1 27	1 41	0 14
3 23	3 39	−0 50	0 46	2 01	2 17	0 16
2 55	2 69	−0 01	0 24	1 98	2 08	0 10
2 26	2 26	−0 23	0 42	1 25	1 15	−0 10
2 84	2 91	0 27	−0 15	2 09	2 06	−0 03
2 88	2 88	−0 27	0 19	1 96	2 20	0 24
2 55	2 63	−0 12	0 21	1 73	1 76	0 03
0 37	0 38	0 30	0 28	0 32	0 38	0 17
		n s	n s	* *	***	n s

phasic deflection of the differential pressure shown in Fig 2 points to the fact that the transmission of pressure were different to the perivesical space and the bladder Since the abdominal cavity contains a variety of tissues liquids and gases and the bladder and the space of Retzius are separated from the abdominal cavity by the *peritoneum* all of which affects the transmission of pressure a rapid pressure change is not equally transmitted to the upper part of the abdominal cavity the space of Retzius and the urinary bladder This fact can explain that the increases in the perivesical and intravesical pressures due to a short rapid intraabdominal pressure increase were smaller than the intragastric pressure increase The return of the differential pressure to the initial value during a maintained strain shows that the time lag was of no importance during a slow pressure change The increases in intravesical and perivesical pressures with change in body posture could therefore be supposed to have been good measures of the increase in intraabdominal pressure even if a small pressure drop between the abdominal cavity and the perivesical space due to a pressure demarcating effect of the peritoneum could not be excluded

That the intragastric pressure was higher in the sitting than in the supine position may be associated with the increased abdominal pressure in the former posture—due to tonus of the musculature of the abdominal wall and pelvic floor to counteract the weight of the viscera It is not particularly surprising that there was no difference between the perivesical and intravesical pressures in either the supine or sitting positions At the time of the investigation the bladder volume was small in all subjects

—eight had no measurable urine volume at the end of the experiment and one had a volume of 50 ml When the bladder volume is small the excess pressure in the bladder is zero (Osborne and Sutherland 1909) That the perivesical and intravesical pressures in the sitting position were higher than the intragastric pressure in this position can be explained by the hydrostatic effect of the viscera

In summary, regardless of body position transmission of pressure changes in the abdominal cavity to the perivesical space and the bladder was incomplete the intragastric pressure change being greater than the perivesical and intravesical changes There was no pressure difference between the latter two points of registration with the bladder almost empty This is in agreement with published observations that in women in the supine position an increase in the intraabdominal pressure was transmitted to the same extent to the bladder and the proximal urethra provided that there was no insufficiency of the pelvic flood with sagging of the bladder and the proximal urethra (Enhorning 1961)

References

BACKMAN K A B VON GARRELTS and R. SUNDBLAD Micturition in normal women Studies of pressure and flow *Acta chir scand* 1966 *132* 403—412

BALMANN U and J SCHMID Ueber den Harnwegsinfektion durch einmaliges Katheterisieren. *Gynaecologia* (Basel) 1967 *164* 221—230

BJERLE P Transmural pressure of the urinary bladder wall *Acta physiol scand* 1974 a. 9? 480—487

BJERLE P Tension stress and modulus of elasticity of the urinary bladder wall *Acta physiol scand* 1974 b 92 488—495

CLARIDGE M Intravesical pressure and outflow resistance during micturition *Acta neurol scand* 1966 *Suppl 20* 95—104

CLARIDGE M and K E D SHUTTLEWORTH The dynamics of obstructed micturition *In est Urol* 1964 *2* 188—199

DENNY BROWN D and G E ROBERTSON On the physiology of micturition *Brain* 1933 *56* 149—190

DESAUTEL R E The causes of catheter induced urinary infections and their prevention *J Urol* (Baltimore) 1969 *101* 757—760

DUBOIS P Ueber den Druck in der Harnblase *Dtsch Arch klin Med* 1876 *17* 158—163

ENHORNING G Simultaneous recording of intravesical and intraurethral pressure *Acta chir scand* 1961 *Suppl 276* 1—68

FRIMODT MOELLER C Clinical urodynamics Methods and results *Scand J Urol Nephrol* 1972 *Suppl 15* 143—155

VON GARRELTS B Intravesical pressure and urinary flow during micturition in normal subjects *Acta chir scand* 1957 a. *114* 49—66

VON GARRELTS B Micturition in urethral stricture *Acta chir scand* 1958 b *114* 40?—4??

GIERUP J Micturition studies in infants and children *Scand J Urol Nephrol* 1970 a *4* 191—207

GIERUP J Micturition studies in infants and children *Scand J Urol Nephrol* 1970 b *Suppl 5* 7—?5

GIERUP J N O ERICSSON and L OKMIAN Micturition studies in infants and children Influence of an indwelling urethral catheter and of rectal pressure recording *Scand J Urol Nephrol* 1970 *4* 20?—215

GLEASON D M and J K LATTIMER The interpretation of voiding pressure *J Urol* (Baltimore) 1964 *91* 156—160

GUYTON A C H I GRANGER and A E TAYLOR Interstitial fluid pressure *Physiol Rev* 1971 *51* 527—563

JUUL JENSEN P Neurological bladder dysfunction *Acta neurol scand* 19?? *38* 113—13?

KASS E H and H S SOSSEN Prevention of infection of the urinary tract in the presence of indwelling catheters *J Amer med Ass* 1959 *169* 1181—1183

KOESTER N and P MADSEN The intragastric pressure before and immediately after truncal vagotomy *Scand J Gastroent* 1970 5 381—383

MOSSO M M and P PELLACANI Sur les fonctions de la vessie *Arch de Biol* (Liege) 1882 *I* 97—128

NYSTROM K P BJERLE and B LINDQVIST Suprapubic catheterization of the urinary bladder as a diagnostic procedure *Scand J Urol Nephrol* 1973 7 160—163

OSBORNE J N and W SUTHERLAND The elasticity of rubber balloons and hollow viscera. *Proc roy Soc B* 1909 *81* 485—499

ROWAN D W T MILLAR and S ALEXANDER An investigation of bladder urethral function by pressure flow studies *Bio med Engng* 1972 7 304—308

RUSHMER R F The nature of intraperitoneal and intrarectal pressure. *Amer J Physiol* 1946 *147* 242—249

SELDINGER S I Catheter replacement of the needle in percutaneous arteriography A new techniqu *Acta radiol* (Stockh) 1953 *39* 368—376

SMITH J C D EDWARDS and G H BRYANT A practical method of measuring urethral resistance to micturition *Brit J Urol* 1966 *35* 542—546

SUNDBLAD R. Urinary bladder dynamics in women *Scand J Urol Nephrol* 1971 *Suppl 6* P 13

SUSSET J H RABINOVITCH and K MACKINNON Parameters of micturition clinical study *J Urol* (Baltimore) 1965 *94* 113—121

SWEDISH STANDARDIZATION BOARD *Swedish standard* 1971 SIS 01 6146

WARRELL D W B W WATSON and T SHELLEY Intravesical pressure measurement in women during movement using a radio-pill and an air probe *J Obstet Gynaec Brit Emp* 1963 *70* 959—967

WISE H M M MANY W C BIRTWELL T B EVRICK and M MAGUIRE Measurement of urethral resistance *Invest Urol* 1968 5 539—551

Acta physiol scand 1974 92 474—479

From the Departments of Clinical Physiology and Gynaecology University of Umeå Sweden

Intra- and Extraperitoneal Pressures

By

Per Bjerle and Bo Sandstrom

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Abstract

Bjerle P *Intra and extraperitoneal pressures* Acta physiol scand 1974 92 474—479

In order to determine the reliability of various methods of recording intraabdominal pressure seven healthy women were investigated in connection with routine laparoscopy Intraabdominal pressure was measured through a trochar in the gas-filled abdomen Intragastric pressure was measured with an air filled balloon perivesical pressure through a catheter in the space of Retzius and intravesical pressure through a Foley catheter introduced via the urethra. With the urinary bladder empty intraabdominal perivesical and intravesical pressures did not differ The intragastric pressure was somewhat higher than the other three pressures possibly due to hydrostatic pressure effects from the viscera. In conclusion if the bladder is empty perivesical as well as intravesical pressure registration appears to be a useful method of fairly closely following the intraabdominal pressure in the supine position without perforating the peritoneum

The pressure in the abdominal cavity varies considerably between different individuals and in different postures (Mengert and Murphy 1933 Drye 1948 von Garrelts 1957 Bjerle 1974) It increases with inspiration and decreases with expiration (Drye 1948) but is otherwise fairly constant in a given person in a given position (von Garrelts 1957) In the supine position the pressure is approximately the same in the cranial and caudal parts of the cavity but as the angle of the body to the horizontal plane increases with head up the pressure at a given point in the cavity is said to increase in proportion to the perpendicular distance from the point of measurement to the dome of the diaphragm (Overholt 1931 Lam 1939 Drye 1948 Warrell *et al* 1963) This increase is a pressure effect due to the weight of the abdominal contents (Mengert and Murphy 1933 Lam 1939 Rushmer 1946)

The methods used to register intraabdominal pressure have certain limitations Recordings made through the medium of air transmitted from the abdominal cavity via a needle (Wagoner 1926) or catheter (Overholt 1931) inserted through the abdominal wall are easily disturbed if the needle or catheter becomes occluded by liquid or other contents of the cavity If an air filled balloon placed in the abdominal cavity by a surgical procedure is used (Lam 1939 Rushmer 1946 Drye

1948) incomplete evacuation of the air that collects in the cavity at laparotomy can give an erroneous pressure level Recording from the rectum by means of a radio transmitter (Wise 1968 open catheter Claridge and Shuttleworth 1964 Claridge 1966 Frimodt Moeller 1972) or balloon filled with air (Denny Brown and Robertsson 1933 Lam 1939 Rushmer 1946 Drye 1948 Gierup *et al* 1970) or liquid (Susset *et al* 1965 Wise *et al* 1948) does not permit accurate quantification because it is difficult to know whether the registered pressures are representative for the whole rectum The tonus of the bowel (Denny Brown and Robertson 1933 Rushmer 1946) and the presence of faeces can give rise to erroneous pressures If the intra gastric pressure is used as a measure of the intraabdominal pressure (von Garrelts 1957 Backman *et al* 1966 Sundblad 1971) it must be borne in mind that the tonus of the stomach can produce falsely high pressures

It was deemed of interest to determine the reliability of various indirect methods of recording the intraabdominal pressure especially that of the method of measuring perivesical pressure described by one of the authors (Bjerle) To this end the intraabdominal pressure has been compared with the results of perivesical intragastric and intravesical pressure recordings

Material

The material for this study consisted of seven women aged 18—38 years average age 29 years admitted to the Department of Gynaecology Umea University Hospital for laparoscopy in connection with investigation of sterility Apart from being possibly sterile all were in good health and none had symptoms of disturbed micturition The patients were told how the trial was to be conducted and gave their full consent.

Methods

A Teflon catheter outer diameter 1 6 mm and inner diameter 1 0 mm equipped at the distal and with a thin walled rubber balloon was passed through a nostril into the stomach The balloon which had a capacity of 55 m without pressure increase (Bjerle) was filled with 40 ml air The catheter was then carefully retracted so that the balloon came to be directly below the cardia of the stomach The patient s urinary bladder was emptied via a Foley catheter which was allowed to remain in the bladder The patient was then anesthetized—intubation and manual ventilation The laparoscope was inserted in th midline just below the navel and CO was insufflated When the abdominal cavity was moderately distended with gas the perivesical catheter was applied with the aid of a Seldinger needle and Teflon guide (Bjerle) Through the laparoscope the movements of the catheter could be observed as a slight bulging of the peritoneum In this way it was ascertained that the catheter had not perforated the peritoneum but that its tip lay immediately outside the anterior wall of the urinary bladder The catheter was filled with physiological saline solution until about 0 5 ml had emerged from the tip into the perivesical space A narrow trochar was inserted into the lower part of the abdominal cavity The catheter in the stomach and the trochar were connected via air filled systems and the perivesical catheter and the bladder catheter via liquid filled systems to the pressure receptors and the recorder Further details regarding the apparatus and methodology are given elsewhere (Bjerle) Under laparoscopic control that the tip of the trochar lay in the gas filled part of the abdominal cavity the pressure was recorded During pressure recording it was checked that slight manual pressure applied to the abdominal wall produced a pressure increase at all four points of registration

The intragastric and intraabdominal pressures in relation to atmospheric pressure and the perivesical and intravesical pressure in relation to the 0-reference pressure located in a plane immediately outside the anterior wall of the bladder (Bjerle) were measured on recordings made during expiration The difference between the pressure at the different points of measurement in each individual patient were calculated and the probability that the mean differences in the patient group were significant was calculated by means of Student s t test.

TABLE I Pressure increase in the stomach, abdominal cavity, perivesical space and bladder produced by slight manual pressure applied to the abdominal wall. 7 women studied in the supine position in connection with laparoscopy shortly after the start of CO_2 insufflation

Patient	Age years	Height cm	Weight kg	Pressure kPa				Pressure in per cent of abdominal pressure			
				Gastric P_G	Abdominal P_A	Perivesical P_P	Bladder P_B	P_G	P_A	P_P	P_B
A R	34	173	59	1.10	1.13	1.04	1.06	97	100	92	94
G E	38	160	58	0.67	0.67	0.53	0.53	100	100	79	79
E H	32	159	59	1.40	1.20	1.22	1.27	117	100	102	107
Å E	18	173	58	1.06	1.01	1.00	1.00	105	100	99	99
D M	18	163	52	0.73	0.56	0.47	0.47	130	100	84	84
G F	27	159	55	1.06	0.85	0.89	0.98	125	100	105	115
R P	36	150	49	1.50	1.52	1.18	1.29	99	100	78	85
Mean	29	162	56	1.07*	1.00	0.90 n.s.	0.91 n.s.				

* = 0.05 > p > 0.01, ns = p > 0.05, where p is the probability that the difference between the pressure and abdominal pressure is caused by random factors

Results

Fig. 1 shows the pressure recording from one patient. The variations with breathing are clearly seen, as is the change in pressure produced by slight pressure to the abdominal wall at the epigastrium. The pressure values are given in Table I and II.

Pressure changes are shown in Table I. Abdominal pressure increase produced by slight manual pressure applied to the abdominal wall caused a pressure increase in

TABLE II Pressure in the stomach and abdominal cavity in relation to atmospheric pressure and perivesical and intravesical pressure in relation to 0-reference pressure in a plane immediately outside the anterior wall of the bladder. Seven women studied in connection with laparoscopy shortly after the start of CO_2 insufflation

Patient	Needle depth cm	Pressure kPa				Difference					
		Gastric P_G	Abdominal P_A	Perivesical P_P	Bladder P_B	$P_A - P_G$	$P_P - P_B$	$P_A - P_P$	$P_A - P_B$	$P_G - P_P$	$P_G - P_B$
A.R	4.0	0.86	0.64	0.98	0.57	−0.22	0.41	−0.34	0.07	−0.12	0.29
C E	4.0	1.06	0.80	0.45	0.52	−0.26	−0.07	0.35	0.28	0.61	0.54
E H	4.0	1.20	0.92	0.72	0.71	−0.28	−0.01	0.20	0.21	0.48	0.44
Å E	4.6	2.06	1.74	1.85	1.32	−0.32	0.53	−0.11	0.42	0.21	0.74
D M	6.4	1.86	1.48	1.45	1.32	−0.38	0.13	0.03	0.16	0.41	0.54
G F	4.3	0.45	0.41	0.40	0.33	−0.04	0.07	0.01	0.08	0.05	0.1
R P	5.0	1.81	1.28	1.36	1.33	−0.53	0.03	−0.08	−0.05	0.45	0.48
Mean	4.6	1.33	1.04	1.03	0.88	−0.29	0.15	0.01	0.16	0.30	0.45
S D		0.55	0.48	0.[illegible]	0.43	0.15	0.23	0.23	0.21	0.26	0.20

* = 0.05 > p > 0.01, ** = 0.01 > p > 0.001, *** = 0.001 > p, where p is the probability that the difference between two mean pressures is caused by random factors

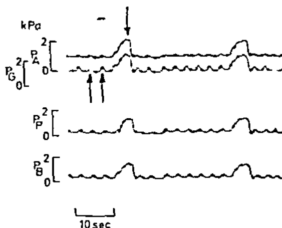


Fig 1 Pressure registration in the supine position in connection with laparoscopy with some CO_2 gas in the abdomen Patient E.H 32 years Abdominal (P_A) gastric (P_G) perivesical (P_P) and bladder (P_B) pressures ↑ denotes pressure increase due to positive pressure ventilation and ↓ due to manual pressure against the abdominal wall

the stomach the perivesical space and the bladder The pressure increase was probably greater in the stomach than in the abdominal cavity In most cases the pressure increase was smaller perivesically and intravesically than intraabdominally but the mean values for the patient group did not differ statistically significantly There was no difference between the perivesical and the intravesical pressure increase

Pressure levels at expiration are shown in Table II Intragastric pressure was higher than intraabdominal pressure and perivesical pressure was equal to intravesical pressure To compare pressures when different reference levels were used assumptions must be made If it is assumed that the lower limit of CO_2 gas was at the level of the upper margin of the urinary bladder which was the 0 reference level for perivesical and intravesical pressures the following applies Intraabdominal pressure did not differ from perivesical and probably not from intravesical pressure intragastric pressure was higher than perivesical and intravesical pressures

Discussion

It has earlier been shown that rapid abdominal pressure changes produced by straining or coughing are not equally transmitted to the stomach and to the bladder and its vicinity probably due to inhomogeneity of the abdominal contents (Bjerle) This mechanism could also partly explain the individual differences in pressure increase perivesically intravesically and intraabdominally when slight manual pressure was applied to the abdomen between the navel and the sternum The greater pressure increase in the stomach could be caused by a compression and deformation of the stomach when pressure was applied

The pressure levels at the 4 points of measurement showed a wide range depending on the intentionally varying degrees of CO_2 insufflation in different patients Different abdominal pressures were wanted since it was the differences between the

pressures at the different points of measurement which were of importance for the goal of the study, which was to compare intragastric, perivesical and intravesical pressures with varying intraabdominal pressure

Following insufflation there was no sharp boundary between CO_2 gas and the abdominal viscera As judged both by laparoscopic findings and by the external configuration of the abdomen insufflation mainly lifted the anterior abdominal wall from a position in which its inner limit in all patients was in the same horizontal plane as the anterior wall of the bladder It was therefore reasonable when calculating pressure differences to use this plane as the 0 reference level for the recordings in which liquid transmission was used

That the perivesical and intravesical pressure levels did not differ from the intraabdominal pressure level with the bladder empty, means that they were adequate expressions of the pressure in the abdominal cavity The variations in pressure due to breathing also support this conclusion as does the fact that manual pressure to the abdominal wall caused a pressure increase at all four points of measurement That the pressure in the stomach was higher than the intraabdominal perivesical and intravesical pressures contrary to earlier report (Bjerle) could be explained by the fact that becau e the stomach was empty the registering balloon was situated somewhat more posterior than the bladder and the lower limit of CO gas The higher pressure therefore need not be due to a transmural pressure difference

No complications due to the perivesical catheter were encountered With the reservation that differences may exist not shown statistically due to the small material perivesical and intravesical pressure registration with the bladder almost empty appears to be a simple method of fairly closely following the pressure in the abdomen without perforating the peritoneum

References

BACKMAN K A B VON GARRELTS and R SUNDBLAD Micturition in normal women Studies of pressure and flow *Acta chir scand* 1966 *132* 403—412

BJERLE P Relationship between perivesical and intravesical urinary bladder pressure and intragastric pressure *Acta physiol scand* 1974 *92* 465—473

CLARIDGE M Intravesical pressure and outflow resistance during micturition *Acta neurol scand* 1966 *42* Suppl 20 95—104

CLARIDGE M and K E D SHUTTLEWORTH The dynamics of obstructed micturition *Invest Urol* 1964 2 2 188—199

DENNY BROWN D and G E ROBERTSON On the physiology of micturition *Brain* 1933 *56* 149—190

DRYE J C Intraperitoneal pressure in the human *Surg Gynec Obstet* 1948 *87* 4 —475

FRIMODT MOLLER C Clinical urodynamics Methods and results *Scand J Urol Nephrol* 1972 Suppl 15 143—155

VON GARRELTS B Intravesical prssure and urinary flow during micturition in normal subjects *Acta chir scand* 1957 *114* 49 –66

GIERUP J N O ERICSSON and L OKMIAN Micturition studies in infants and children Influence of an indwelling urethral catheter and of rectal pressure recording *Scand J Urol Nephrol* 1970 *4* 209—215

LAM C R Intra abdominal pressure A critical review and an experimental study *Arch Surg* 1939 *39* 1006—1015

MENGERT W F and D P MURPHY Intraabdominal pressures *Surg Gynec Obstet* 1933 *57* 745—751
OVERHOLT R H Intraperitoneal pressure *Arch Surg* 1931 *22* 691—703
RUSHMER, R F The nature of intraperitoneal and intrarectal pressures *Amer J Physiol* 1946 *147* 242—249
SUNDBLAD R Urinary bladder dynamics in women *Scand J Urol Nephrol* 1971 *Suppl 6* 5—51
SUSSET J H RABINOVITCH and K MACKINNON Parameters of micturition clinical study *J Urol* 1965 *94* 113—121
WAGONER G W Studies on intraabdominal pressure I Negative intraabdominal pressure as a normal condition *Amer J med Sci* 1926 *171* 697—707
WARRELL, D W B W WATSON and T SHELLEY Intravesical pressure measurement in women during movement using a radio-pill and an air probe *J Obstet Gynaec Brit Emp* 1963 *70* 959—967
WISE H M M MANY W C BIRTWELL T B EYRICK and M MAGUIRE Measurement of urethral resistance *Invest Urol* 1968 *5* 539—551

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From the Department of Clinical Physiology University of Umeå Sweden

Transmural Pressure of the Urinary Bladder Wall

By

PER BJERLE

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Abstract

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Intragastric perivesical and intravesical pressures were registered during slow filling of the urinary bladder in nine healthy men in the supine and sitting positions Intragastric pressure was fairly constant in both positions but intravesical pressure rose and there was also a slight rise of perivesical pressure In both body positions transmural pressure i.e the pressure difference between the inside and the outside of the bladder rose during bladder filling In both body positions at bladder volumes ⩽ 300 ml in this material transmural pressure determined as the difference between intravesical and perivesical pressures did not differ statistically significantly from transmural pressure calculated as the difference of intravesical and intragastric pressures minus a hydrostatic component. At maximal bladder volumes transmural pressure determined in the first way was statistically significantly lower than that calculated in the second way In the supine position the transmural pressure at bladder volume 300 ml calculated as the difference of intravesical and perivesical pressures was lower than in the upright position

Knowledge of the pressure relationships within and outside the urinary bladder is a prerequisite for correct evaluation of its functional state Obviously the degree of distension and/or contraction of the bladder wall produces variations in the intravesical pressure and the latter is modified by the pressure outside the bladder (Dubois 1876 Bjerle 1974 a) Among the factors that determine the intraabdominal extravesical pressure are the tonus or state of contraction of the abdominal wall and the diaphragm Emerson 1911 Mengert and Murphy 1933 Drye 1948) the weight of the contents of the abdominal cavity (Lam 1939 Rushmer 1946) and not least the body posture (Mengert and Murphy 1933 Drye 1948 Bjerle 1974 a)

The intravesical pressure can be measured by means of a urethral catheter (Dubois 1876 Mosso and Pellacani 1882 Denny Brown and Robertson 1933 von Garrelts 1957 Enhörning 1961 Gierup *et al* 1970 through a catheter inserted suprapubically (Sandow *et al* 1959 Claridge and Shuttleworth 1964 Susset *et al* 1965 Hin… 1968 Gierup *et al* 1969 Frimodt Møller 1972 Rowan *et al* 1972) or by means of a manometer freely mobile within the bladder (Gleason and Lattimer 19… Warrell *et al* 1963) The intraabdominal pressure can be measured via a balloon

TABLE I Bladder volume at slight and urgent desire to void in the supine and sitting positions urethral resistance and residual urine in the supine and standing positions in nine healthy men The values within brackets are the bladder volumes at initiation of micturition The bladder volume at urgent desire to void was obtained as the sum of voided volume and residual volume In the supine position two subjects R L and H O were unable to void and two subjects G P and S Å A could void only with moderately filled bladder in these cases the volume at urgent desire to void was fully obtained by emptying the bladder through the suprapubic catheters If the subject micturated twice the minimal urethral resistance has been used for calculation of the mean

		Supine				Sitting		Standing	
Subject	Age years	Bladder volume at slight desire to void ml	Bladder volume at urgent desire to void ml	Urethral resistance Pa s ml⁻	Residual urine ml	Bladder volume at slight desire to void ml	Bladder volume at urgent desire to void ml	Urethral resistance Pa s ml	Residual urine ml
O B	42	350	480	Not requested to void		150	470	18 0 (470) 16 5 (480)	0 0
G P	38	260	710	34 0 (220)	0	500	810	12 3 (710) 9 0 (810)	10 0
R L	31	340	440	Unable to void		270	365	35 3 (365)	0
S Å A	42	365	560	43 8 (300)	0	350	540	72 8 (540)	25
K A	58	320	530	8 2 (500)	30	360	660	3 8 (660)	0
H O	36	170	375	Unable to void		170	355	41 5 (355)	0
P A L	30	170	315	13 6 (315)	0	175	345	22 2 (345)	0
K E B	33	350	965	25 7 (730) 51 8 (965)	210 375	380	935	31 6 (935)	0
G G	42	395	590	18 7 (590)	175	275	565	22 1 (315) 17 7 (565)	10 0
Mean	39	302	552			292	555	27 8	
S D	8 5	83	195			116	212	20 8	

placed in the abdominal cavity by a surgical procedure (Lam 1939 Rushmer 1946 Drye 1948) or by means of a needle (Wagoner 1926) or a catheter (Overholt 1931) inserted through the abdominal wall The pressure in the upper part of the abdominal cavity can be determined by registering the pressure in the stomach (von Garrelts 1957 Backman 1966 Sundblad 1971) Pressure recordings made with a catheter (Claridge and Shuttleworth 1964 Claridge 1966 Frimodt Moller 1972) or balloon (Denny Brown and Robertson 1933 Lam 1939 Rushmer 1946, Drye 1948 Susset *et al* 1965 Wise 1968 Gierup 1970 Gierup *et al* 1970) in the rectum can supply information on the pressure in the lower part of the abdomen

The transvesical pressure or excess pressure in the bladder has been estimated by subtracting the intraabdominal pressure from the intravesical pressure (Gleason *et al* 1964 Backman *et al* 1966 Sundblad 1971 Frimodt Moller 1972) However uncertainty as to the magnitude of the hydrostatic component to be added to the intragastric pressure (Sundblad 1971) or the effect of faeces and intestinal tonus on

the rectal pressure (Denny Brown and Robertson 1933, Rushmer 1946) make such subtractions unreliable or subject to systematic error (Sundblad 1971)

By means of a catheter applied perivesically, however, it is possible to determine the extravesical pressure fairly accurately in both the supine (Bjerle and Sandstrom 1974) and the upright (Bjerle 1974 a) position Previous work (Bjerle 1974 a Bjerle and Sandstrom 1974) has shown that in both the supine and erect postures, provided the bladder is empty or nearly so, extravesical pressure does not differ from intravesical pressure *i e* transmural pressure is zero

The aim of the present study was to determine the perivesical and intravesical pressures and thereby also the transmural pressure in men during filling of the urinary bladder (cystometry), and to compare the transmural pressure measured in this way with the transmural pressure calculated on the basis of the intravesical and intragastric pressures

Material

The material consisted of nine healthy men with a mean age of 39 (30—58) years They had never had any disturbance of micturition Data on the material are presented elsewhere (Bjerle 1974 a) and in Table I

Methods

The intragastric pressure was recorded by means of a balloon at the end of a Teflon catheter the perivesical pressure by means of a suprapubic Teflon catheter with its tip in the space of Retzius and the intravesical pressure via a suprapubic Teflon catheter For details of these methods see previous publications (Bjerle 1974 a, Bjerle and Sandstrom 1974) For instillation of lukewarm saline during cystometry and for aspiration of residual urine another suprapubic catheter PE 160 was inserted 0 5 cm lateral to the midline and contralateral to the point of insertion of the intravesical catheter used for pressure recording At micturition the urine was collected in a vessel resting on a steel support connected to a strain gauge recorder The rate of urine flow was obtained by means of a derivation unit The catheters for pressure registration were connected with a recording apparatus consisting of pressure receptors electromanometers and a direct writing ink oscillograph (EMT 34 EMT 31 and Mingograf 81 made by Siemens Elema Stockholm Sweden) Two of the pressure channels could be coupled to a subtraction unit and the resulting pressure difference was registered by the Mingograf writer (Bjerle 1974 a) The writer recorded both the urine volume and the rate of urine flow By means of an amplifier four sequences of events could be transferred from the Mingograf writer to a tape recorder and could subsequently be written out with a reduced time scale The volume displacement of the catheter transducer unit was 1 6 mm³ when exposed to a pressure of 10 kPa. The catheter transducer system gave correct deflections without resonance up to a frequency of about 15 Hz when tested with sine wave pressures The writer produced deflections proportional to applied pressure standards in the range employed

The rate of infusion in cystometry is often disregarded but it is of great importance for the intravesical pressure A high rate of infusion may elicit contraction of the detrusor (Gryn..., 1966) whereas this is difficult to bring about at an infusion rate of 50 ml/min or less When the rate of infusion is low the bladder's viscous properties are not apparent it behaves instead like an elastic body (Apter *et al* 1972 Kondo *et al* 1972) For this reason the low infusion rate 50 ml/min was chosen

The subjects were investigated in the supine position by means of micturition and then cystometry followed by another micturition and in the upright position by micturition standing and then cystometry while seated followed by micturition standing

During cystometry pressures were continuously recorded and during micturition urine volume and rate of urine flow were registered as well After each micturition which took place when the subject experienced an urgent need to void a check was made for the presence of residual urine by suction through the suprapubic bladder catheters Also the

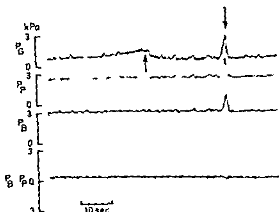


Fig 1 Example of pressure registration in the sitting position Subject G P P_G = intragastric pressure P_P = perivesical pressure P_B = intravesical (bladder) pressure P_B—P_P = transmural pressure ↑ denotes intragastric pressure fluctuations due to contraction of the stomach ↓ denotes pressure fluctuations due to straining

subject in the supine position could not void the bladder was emptied through the supra pubic catheters In no case the volume obtained as the sum of micturated and sucked out volume was less than the infused volume In most cases it was larger and thus no great perivesical leakage could have taken place Four of the subjects were first investigated in the supine position and then upright for the other five this order was reversed Urethral resistance at maximal urine flow was calculated as the quotient of intravesical pressure and the square of urine flow (Smith *et al* 1966)

Results

A slight urge to void arose in the supine position at a volume of 302 ± 83 ml mean and S D and in the sitting position at a bladder volume of 292 ± 116 ml The maximal bladder volume *i e* the volume at which the subjects experienced an urgent desire to void was in the supine position 552 ± 195 ml and in the sitting position 555 ± 212 ml Thus the volume at the time of slight and urgent desire to void respectively was the same in both the supine and upright positions (Table I) In both positions all subjects reached bladder volumes of 300 ml or more The means of the intragastric perivesical intravesical and transmural pressures have been calculated for the volume interval 0—300 ml

Intragastric pressure In 7 of the subjects there were fluctuations of the intragastric pressure in both the supine and sitting positions which were unrelated to the perivesical and intravesical pressures (Fig 1) These fluctuations consisted of 30 (15—45) s long humps in the pressure curve exceeding the basal pressure by 0 9 (0 3—2 9) kPa In 2 of the subjects no fluctuations of this type were seen Apart from such fluctuations and apart from small variations with breathing the intragastric pressure was fairly constant for all bladder volumes and virtually the same in the supine and sitting positions (Fig 2 Table II)

Perivesical pressure The perivesical pressure rose during filling of the bladder in the supine position and in the sitting position The pressure increase in the supine position was greater than in the sitting position

Intravesical pressure In the supine and sitting positions the intravesical pressure rose to the same extent during filling of the bladder

TABLE II Mean values of intragastric (P_G) perivesical (P_P) intravesical (P_B) and transmural (P_T) pressures in the supine and sitting positions at bladder volumes 0–300 ml reg = = registered est = estimated P_{BO} and P_{GO} are pressures when the bladder is empty

	Pressure kPa							Pressure increase (0–300 ml) kPa
	Bladder volume							
	0	50	100	150	200	250	300	
Supine								
P_G	0.70	0.67	0.69	0.70	0.71	0.73	0.78	0.08 n.s
P_P	0.81	0.86	0.90	0.95	0.99	1.03	1.09	0.28 ***
P_B	0.87	1.05	1.13	1.21	1.25	1.30	1.37	0.50 **
$P_{Treg} = P_B - P_P$	0.06	0.19	0.23	0.26	0.26	0.27	0.28 (1)	0.22 *
$P_{Test} = P_B - P_G - (P_{BO} - P_{GO})$	0.00	0.21	0.27	0.34	0.37	0.40	0.42 (3)	0.42
Sitting								
P_G	0.91	0.94	0.93	0.89	0.93	0.94	0.91	0
P_P	2.55	2.60	2.62	2.65	2.70	2.72	2.70	0.15
P_B	2.63	2.83	2.95	3.01	3.07	3.12	3.13	0.50
$P_{Treg} = P_B - P_P$	0.08	0.23	0.33	0.36	0.37	0.40	0.43	0.35
$P_{Test} = P_B - P_G - (P_{BO} - P_{GO})$	0.00	0.18	0.30	0.40	0.42	0.46	0.50 (4)	0.50 *

Differences (1) – (2) = 0.14 n.s
(3) – (4) = 0.07 n.s
(5) – (6) = 0.13*
(1) – (3) = –0.15

n.s = $p > 0.05$ * $0.05 > p > 0.01$ ** $= 0.01 > p > 0.001$ *** $= 0.001 > p$ where p = the probability that the difference is caused by random factors

) *Transmural pressure* The transmural pressure was determined in two ways—first directly as the difference between the intravesical pressure P_B and the perivesical pressure P_P registered via the perivesical cathter according to the relationship

$$P_T = P_B - P_P \qquad [1]$$

secondly indirectly based on intravesical and intragastric pressures The following theory was applied

$$P_T = P_B - P_G - (P_{P_O} - P_{G_O}) \qquad [2]$$

where P_G is the intragastric pressure—transient fluctuations excepted—and $(P_{P_O} - P_{G_O})$ is the hydrostatic pressure component with the bladder empty B since the transmural pressure is about zero when the bladder is empty (Bjerle 1974 a) equation [2] can also be written in the following way

$$P_T = P_B - P_G - (P_{B_O} - P_{G_O}) \qquad [3]$$

where P_{P_O} is the pressure in the empty bladder

The transmural pressure according to [1] and [3] is shown in Table II and Fig 3 It rose as the bladder filled At bladder volume 300 ml the pressure calculated according to [1] was lower than the pressure calculated according to [3] but there was no statistically significant difference in this material in either the supine or sitting

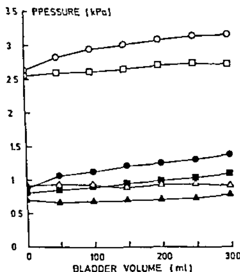


Fig 2

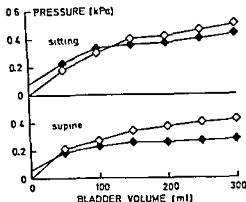


Fig 3

Fig 2 Intragastric (△) perivesical (□) and intravesical (O) end-expiratory pressure levels in the supine (filled symbols) and sitting (open symbols) position in 9 healthy men

Fig 3 Transmural bladder pressure determined as the difference between intra and perivesical pressures (filled symbols) and as the difference between intravesical and intragastric pressures minus the pressure difference between empty bladder and the stomach (open symbols) in 9 healthy men

position for bladder volumes 0—300 ml The mean difference between transmural pressures calculated according to [1] and [3] at maximal bladder volume in each subject was however statistically significant in both the supine and sitting positions In the supine position it was 0 40 kPa and sitting 0 21 kPa In the supine position at bladder volume 300 ml the transmural pressure determined according to [1] was lower than in the sitting position With increasing bladder volumes this difference was even more marked

Discussion

To avoid injury to the bladder an urgent desire to void should always be respected as a sign that the upper limit of the bladder s capacity has been reached (Glahn 1971) Even if further filling was possible the volume at which there was an urgent desire to void was chosen as a measure of the maximal bladder volume That this value for maximal bladder volume is the same in the supine and sitting positions indicates that a mechanism independent of posture elicits the desire to void The intravesical pressure level can thus be excluded as the eliciting factor as it is different in the two positions

The fluctuations in the intragastric pressure in relation to atmospheric pressure that were noted must have been due to contractions of the stomach since they were not registered peri and intravesically They were therefore disregarded in the indirect determination of transmural pressure That the intragastric pressure was th

same in the supine and sitting positions has been reported in a previous publication (Bjerle 1974 a). That it is relatively constant during filling of the bladder is reasonable, since an increase in the volume of the bladder produces a very slight decrease in the volume of the abdominal cavity, such as might be envisaged as increasing the intraabdominal and thereby the intragastric pressure.

The perivesical pressure is naturally greater in the sitting position than when the body is horizontal due to the hydrostatic pressure effect of the viscera. The fact that it is influenced by the degree of filling of the bladder must mean that the pressure in the extraperitoneal space in which the bladder is located does not equilibrate with that of the abdominal cavity due to the demarcating effect of the *peritoneum, but that an increase of the bladder volume encroaches on the volume* of this space and thereby causes an increase in pressure. A volume increase of the smaller space below the peritoneum has hardly any effect on the volume of the abdominal cavity and thus does not result in an intraabdominal pressure increase. The perivesical pressure increased more in the supine than in the sitting position. Perhaps the peritoneum is more stretched in the former position and yields a higher perivesical pressure when this limited space is encroached upon by the bladder.

It was in this investigation impossible to show that there was complete pressure equilibrium in the perivesical space, but the differences that may exist are likely to be small. In any case the measured perivesical pressure is probably the best available measure of the average perivesical pressure when the bladder is not empty. If the pressure measured in the space of Retzius was too low, the transmural pressure would not have been about zero with the bladder empty and if it was too high it would not, with an empty bladder, have been equal to the abdominal pressure, which was reported earlier (Bjerle and Sandstrom 1974).

The intravesical pressure increase was the same in both the body positions but because the perivesical pressure increased more in the supine than in the sitting position, the transmural pressure rose less in the former position than with the subject sitting. For a possible explanation of this fact see Bjerle (1974 b).

The difference between the transmural pressures calculated on the basis of recorded intra- and perivesical pressures [1] and recorded intravesical and calculated perivesical pressure [3] was not statistically significant in this material when the bladder volumes were small, but was so at maximal volumes. Therefore, provided that the pressure fluctuations which were due to contractions of the stomach and which were not transmitted to the urinary bladder were disregarded, determination of the transmural pressure on the basis of the intravesical and intragastric pressure according to [3] may be regarded as approximately equivalent to direct determination according to [1] in healthy men when the bladder volume is < 300 ml.

References

APTER, J. T., P. MASON and C. LANO: Urinary bladder wall dynamics. *Invest. Urol.* 19[illegible] 520—526.

BACKMAN, K. A., B. VON GARRELTS and R. SUNDBLAD: Micturition in normal women. Studies of pressure and flow. *Acta chir. scand.* 1966 132 403—412.

BJERLE P Relationship between perivesical and intravesical urinary bladder pressures and intragastric pressure *Acta physiol scand* 1974 a *92* 465—473
BJERLE P Tension stress and modulus of elasticity of the urinary bladder wall. *Acta physiol scand* 1974 b *92* 488—495
BJERLE P and B SANDSTROM Intra and extraperitoneal pressures *Acta physiol scand* 1974 *92* 474—479
CLARIDGE M Intravesical pressure and outflow resistance during micturition *Acta neurol scand* 1966 *Suppl 20* 95—104
CLARIDGE M and K E D SHUTTLEWORTH The dynamics of obstructed micturition *Invest Urol* 1964 *2* 188—199
DENNY BROWN D and G E ROBERTSON On the physiology of micturition *Brain* 1933 *56* 149—190
DRYE J C Intraperitoneal pressure in the human *Surg Gynec Obstet* 1948 *87* 472—475
DUBOIS P Ueber den Druck in der Harnblase *Dtsch Arch klin Med* 1876 *17* 148—163
EMERSON H Intra abdominal pressure *Arch int Med* 1911 *6* 754—784
ENHORNING G Simultaneous recording of intravesical and intraurethral pressure *Acta chir scand* 1961 *Suppl 276* 1—68
FRIMODT MOELLER C Clinical urodynamics Methods and results *Scand J Urol Nephrol* 1972 *Suppl 15* 143—155
VON GARRELTS B Intravesical pressure and urinary flow during micturition in normal subjects *Acta chir scand* 1957 *CXII* 49—66
GIERUP J Micturition studies in infants and children *Scand J Urol Nephrol* 1970 *4* 191—207
GIERUP J N O ERICSSON and L OKMIAN Micturition studies in infants and children *Scand J Urol Nephrol* 1969 *3* 1—8
GIERUP J N O ERICSSON and L OKMIAN Micturition studies in infants and children Influence of an indwelling urethral catheter and of rectal pressure recording *Scand J Urol Nephrol* 1970 *4* 209—215
GLAHN B E Overdistension of blaeren og principper for kateter à démeure regime *Nord Med* 1971 *4* 128—128
GLEASON D M and J K LATTIMER, A miniature radio transmitter which is inserted into the bladder and which records voiding pressure *J Urol* 1962 *87* 507—509
GLEASON D M and J K LATTIMER The interpretation of voiding pressure *J Urol* (Baltimore) 1964 *91* 156—160
GRYNDERUP V Double cystometry in the uninhibited neurogenic bladder *Acta neurol scand* 1966 *Suppl 20* 67—77
KONDO A J G SUSSET and J LEFAIVRE Viscoelastic properties of bladder I Mechanical model and its mathematical analysis *Invest Urol* 1972 *10* 154—163
LAM C R Intra abdominal pressure A critical review and an experimental study *Arch Surg* 1939 *39* 1006—1015
MENGERT W F and D P MURPHY Intra abdominal pressures created by voluntary muscular effort *Surg Gynec Obstet* 1933 *LVII* 745—751
MOSSO A and P PELLACANI Sur les fonctions de la vessie *Arch de Biol* (Liege) 1882 *1* 97—128
OVERHOLT R H Intraperitoneal pressure. *Arch Surg* 1931 *22* 691—703
ROWAN D W T MILLAR and S ALEXANDER An investigation of bladder urethral function by pressure flow studies *Bio Med Engng* 1972 *7* 304—308
RUSHMER, R F The nature of intraperitoneal and intrarectal pressures *Amer J Physiol* 1946 *147* 242—249
SANDOE E E J BRYNDORF and T GERTZ Cystometry A new technique applying a percutaneously inserted catheter in the bladder *Dan med Bull* 1959 *6* 194—197
SMITH J C D EDWARDS and G H BRYANT A practical method of measuring urethral resistance to micturition. *Brit J Urol* 1966 *35* 542—546
SUSSET J H RABINOVITCH and K MACKINNON Parameters of micturition clinical study *J Urol* (Baltimore) 1965 *94* 113—121
SUNDBLAD R. Urinary bladder dynamics in women *Scand J Urol Nephrol* 1971 *Suppl 6* 5—51
WAGONER, G W Studies on intra abdominal pressure I Negative intra abdominal pressure as a normal condition. *Amer J Med Sci* 19 6 *171* 697—707
WARRELL D W B W WATSON and T SHELLEY Intravesical pressure measurement in women during movement using a radio-pill and an air probe. *J Obstet Gynaec Brit Emp* 1963 *70* 959—967
WISE H et al Measurement of urethral resistance *Invest Urol.* 1968 *5* 539—551

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From the Department of Clinical Physiology University of Umeå Sweden

Tension, Stress and Modulus of Elasticity of the Urinary Bladder Wall

By

PER BJERLE

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Abstract

BJERLE P *Tension stress and modulus of elasticity of the urinary bladder wall* Acta physiol scand 1974 92 488—495

Transmural bladder pressure i e the difference between the intravesical and perivesical pressures was continuously registered during slow filling of the bladder in nine healthy men On the basis of the pressure thus obtained the surface tension of the bladder was calculated From the latter and from the assumed thickness of the bladder wall the normal stress in the wall and a differential modulus of elasticity were calculated The surface tension the normal stress and the differential modulus of elasticity increased markedly as the radius of the bladder increased The logarithm of the maximal differential modulus of elasticity registered was linearly correlated to the bladder radius This relationship makes it possible to predict in normal subjects the differential modulus of elasticity from the bladder radius and to obtain information regarding possible abnormalities in the mechanical properties of the bladder wall

The urinary bladder can be regarded as a spherical bag composed of muscle and connective tissue elements located in the lower part of the abdomen and separated from the abdominal cavity by the peritoneum As the bladder functions alternatively as a reservoir and as an excretory organ the intravesical pressure undergoes periodic variations In addition the intraabdominal pressure is transmitted to the bladder and the proximal urethra (Enhorning 1961) Rapid pressure changes are not fully transmitted to the perivesical space and the bladder during sustained intra-abdominal pressure change the pressure equilibrium is more complete between the abdominal cavity and the region immediately surrounding the bladder although a small pressure difference may still exist (Bjerle 1974 a Bjerle 1974 b Bjerle and Sandström 1974) During the phase of bladder contraction to expel the urine stored there is a marked rise in intravesical pressure due to contraction of the detrusor For this contraction to occur there must be a functioning parasympathetic cholinergic innervation (La Grange 1971) and a functioning detrusor muscle During the phase of relaxation in which the bladder fills with urine from the ureters the intravesical pressure is low and shows very little increase with increasing bladder volume

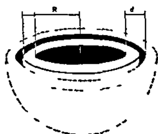


Fig 1 Schematic drawing of the urinary bladder at two degrees of expansion and the variables used

(Langley and Whiteside 1951 Juul Jensen 1962 Glahn 1970) Animal experiments and studies of humans with beta receptor blockade ganglionic blockade destruction of the spinal cord and denervation of the bladder have shown that apart from a certain relaxing function of the sympathetic system the innervation of the bladder is of no importance during this phase (Nesbit and Lapides 1948 Langley and Whiteside 1951 Tang and Ruch 1955 Plum 1960 Edvardsen 1966 La Grange 1971) The intravesical pressure during this phase reflects the physical state of the bladder (Tang and Ruch 1955) and is determined by its viscoelastic properties (Hinman and Miller 1964) If the rate of filling of the bladder is low or if the bladder is allowed to accomodate after rapid filling its viscous properties become negligible and its wall behaves like an elastic body (Apter 1972 Kondo 1972) Of course it is the excess pressure in the bladder in relation to its surroundings which is of interest in this context By registering the perivesical pressure (Bjerle 1974 a) together with the intravesical pressure the transmural pressure during the phase of bladder filling can be determined (Bjerle 1974 b) It is approximately 0 kPa when the bladder is empty and rises to about 0.3 kPa at a bladder volume of 300 ml in the supine position and 0 4 kPa at a bladder volume of 300 ml in the sitting position

If the bladder is assumed to be spherical (Fig 1) and the law of Laplace

$$\sigma = \frac{P\ R}{2} \qquad [1]$$

is applied (Susset 1971) in which σ_1 is surface tension P transmural pressure and R bladder radius it follows that at a constant transmural pressure the surface tension is higher the greater the bladder radius

The surface tension of the bladder wall is related to the total force acting in a diametrical cross-section of the bladder according to the equation

$$\sigma_t = \frac{S}{2\ \pi\ R} \qquad [?]$$

where S is the total force and P the bladder radius. The relationship between S and R can also be expressed

$$\sigma = \frac{S}{2\ \pi\ R\ r} \qquad [3]$$

where σ is the normal stress of the bladder wall and r the thickness of the wall From equations [2] and [3] follows

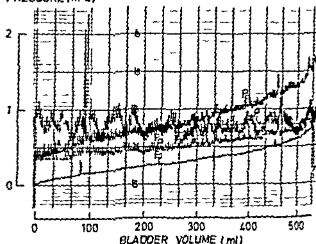


Fig 2 Pressure registration in the supine position during filling of the bladder with a rate of 50 ml/min in subject K.A P_G = gastric pressure P_P = perivesical pressure P_B = intravesical pressure and P_T ($= P_B - P_P$) – transmural pressure The pressures fluctuated due to respiration (P_P and P_B) and due to respiration combined with contraction of the stomach (P_G)

$$\sigma = \frac{\sigma_t}{r} \qquad (2)$$

Calculation of tension of the urinary bladder wall according to equation (1) does not take the variation of the bladder thickness into account. As the thickness of the bladder wall t diminishes with increasing bladder radius R according to

$$t = \frac{W}{4 \pi R} \qquad (3)$$

and assuming the muscle volume W to be constant the normal stress is a better measure of the elastic properties of the wall To the author's knowledge the relationship in equation (3) has been taken into consideration in only one published study (Sundblad 1971)

There seems to have been no investigation of the tension and stress in the bladder wall during the filling phase It was deemed desirable to also study this phase, which is far different from the phase of micturition and to derive means of expressing the mechanical properties of the bladder wall

Material

The material consisted of nine healthy men without any history or symptoms pertaining to the bladder and urinary tract aged 30–58 years mean age 39 years. Data on this group of subjects are presented elsewhere Bjerle 1974 a)

Methods

Theory By definition the relationship between the relative elongation and the stress of a body is

$$E_0 = \frac{\sigma}{\varepsilon} \qquad (4)$$

where E_0 is the modulus of elasticity in N/m or Pa, σ normal stress in N/m and ε relative elongation (Beckman 1964) If the urinary bladder is assumed to be a sphere with radius R the relative elongation is

$$\varepsilon = \frac{dR}{R} \quad dR \ll R \qquad [7]$$

where dR is the change in radius and R the original radius If equations [4] and [7] are substituted in equation [6] the following expression for the modulus of elasticity is obtained

$$E_0 \quad \frac{\sigma}{r} \frac{R}{dR} \qquad [8]$$

Since the surface tension σ_1 is dependent on the bladder radius according to equation [1] equation [8] may be written

$$E_0 = \frac{\sigma (R)}{r} \frac{R}{dR} \qquad [9]$$

where σ_1 (R) indicates that σ_1 is a function of R This means that E_0 will assume different values during filling of the bladder due to the non linear relationship between σ_1 and R according to [1] since P is positively correlated to R (*cf* Fig 2) Differentiation of equation [9] gives

$$dE_0 = \frac{d\sigma}{dR} \quad \frac{R}{r} + 2\sigma_t = E + 2\sigma \qquad [10]$$

considering the relative elongation $\frac{dR}{R}$ as a constant. In the experimental situation it turns out that 2 σ_2 is approximately constant compared to E Furthermore the measurement of σ_1 implies a correct determination of the transmural pressure On the other hand the measurement of

$$E = \frac{d\sigma_t}{dR} \quad \frac{R}{r}$$

implies just determination of the derivative of the radial dependence of σ_1 which is much easier to do in clinical practice Therefore the term 2 σ_2 is omitted and the following modification of equation [9] is introduced

$$E = \frac{d\sigma}{dR} \quad \frac{R}{r} \qquad [11]$$

where E is a differential modulus of elasticity which is a measure of the elastic property of the bladder at the radius R The factor $\frac{d\sigma}{dP}$ is available from an empirically obtained curve relating surface tension to bladder radius The bladder radius R is calculated from the bladder volume (= urine volume) The thickness of the bladder r can be estimated according to Sundblad (1971)

Procedure and apparatus The methods and apparatus used to register pressures are described in detail elsewhere (Bjerle 1974 a and b) Briefly two catheters were inserted suprapubically The tip of one was positioned within the bladder and that of the other immediately outside the bladder wall The differential pressure *i e* the transmural pressure was continuously recorded as the bladder was slowly filled by means of a third suprapubic catheter (Fig 2) The transmural pressure was measured at 50 ml intervals and the surface tension was calculated according to equation [1] The slope of the curve of tension/radius was obtained from the quotient of tension increase and increase in radius for every 50 ml increase in bladder volume except for the last increment, which in most cases was < 50 ml For each increment the mean of the endpoint radii was used as a measure of R This mean was also used to calculate the bladder wall thickness for each increment according to Sundblad (1971)

Results

The surface tension of the bladder wall in relation to the radius of the bladder is shown in Fig 3 In most of the experimental subjects the surface tension fluctuated during filling of the bladder In two of them the surface tension was fairly constant in the remaining seven it increased with increasing bladder radius Fig 4 shows the mean normal stress of the bladder wall in relation to the bladder rad

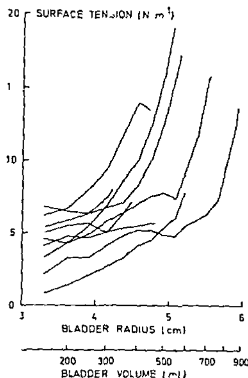


Fig 3

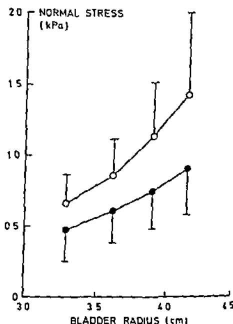


Fig 4

Fig 3 Relationship between the surface tension and bladder radius in the supine position in nine healthy men

Fig 4 Relationship between the normal stress mean ±1 SD and bladder radius in the supine (●) and sitting (○) positions in 9 healthy men

bladder radius of 3 29 cm it was 474 (±222) Pa in the supine position and 661 (±209) [mean and SD] in the sitting position at a bladder radius of 4 15 cm it was 905 (±322) Pa supine and 1429 (±577) Pa when sitting The calculated bladder wall stress was thus greater in the sitting position than with the body supine

The relationship between the differential modulus of elasticity of the bladder wall and the bladder radius is shown in Fig 5 The modulus fluctuated for all subjects and in five assumed a negative value at some time In three subjects the modulus of elasticity reached a maximum before maximal bladder volume was attained in six subjects the modulus of elasticity was highest at maximal bladder volume With the exception of the interval in which the modulus of elasticity decreased it appeared to increase exponentially as the radius increased For this reason the maximal differential modulus of elasticity obtained for each subject was expressed as a logarithm and these logarithms were plotted against the bladder radii The resulting points could with confidence be fitted to a straight line (correlation coefficient 0 93 $p < 0.001$) (Fig 6)

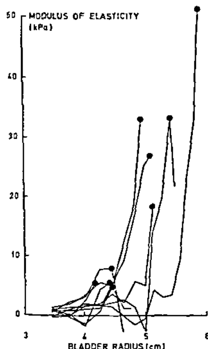


Fig 5

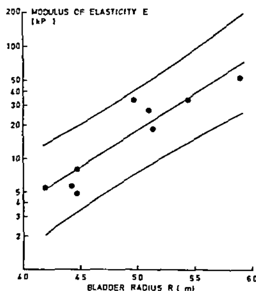


Fig 6

Fig 5 Relationship between the differential modulus of elasticity and bladder radius in the supine position in 9 healthy men ● = maximal value f the modulus

Fig 6 The logarithm of the maximal differential modulus of elasticity in nine healthy men related to the bladder rad us The equation of the regression line calculated as product moment correlation according to the method of least squares is lnE = 1 54 R—4 86 Correlation coefficient (r) = 0 93 0 001 > p The curved lines are the limits of the 95 % confidence interval for prediction of E when R is known

Discussion

The calculations of surface tension normal stress and modulus of elasticity are based on the assumption that the urinary bladder is spherical in shape This is not true if the bladder volume is small and for this reason calculations have been performed only for volumes of 150 ml or more Even at larger bladder volumes the assumption that the bladder is a sphere may not be valid in the sitting position for the following reasons Were the bladder freely movable in the abdomen it would assume a spherical shape due to the tension in the wall However the bladder is anchored below by its continuity with the urethra and anteriorly be the umbilical ligament These points of attachment to the lower part of the abdomen together with the downward pressure of the viscera flatten the bladder when the body is in the sitting position In the supine position the bladder is 'hanging' down from its attachments into the visceral package and is subjected to fairly low uniform pressure on all sides it is thus able to assume an almost spherical form Examination in connection

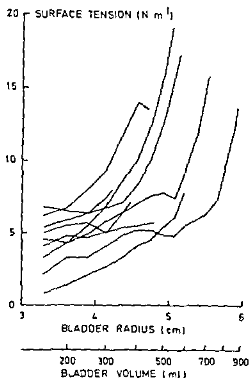


Fig 3

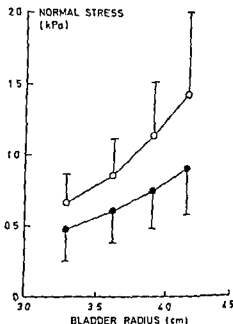


Fig 4

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EDVARDSEN P Changes in urinary bladder motility following lesions in the nervous system in cats *Acta neurol scand* 1966 *42* 25—31
ENHORNING G Simultaneous recording of intravesical and intraurethral pressure *Acta chir scand* 1961 *Suppl 276* 1—68
GLAHN B F Neurogenic bladder diagnosed pharmacologically on the basis of denervation supersensitivity *Scand J Urol Nephrol* 1970 *4* 13—24
GLAHN B E Overdistension af blaeren og principper for kateter à demeure regime *Nord Med* 1971 *28* 128—128
HINMAN F and E R MILLER Mural tension in vesical disorders and urethral reflux *J Urol* (Baltimore) 1964 *91* 33—40
JUUL JENSEN P Neurological bladder dysfunction. *Acta neurol scand* 1962 *38* 113—130
KONDO A J G SUSSET and J LEFAIVRE Viscoelastic properties of bladder I Mechanical model and its mathematical analysis *Invest Urol* 1972 *10* 154—163
LA GRANGE R G Peripheral autonomic regulation of the canine urinary bladder *Invest Urol* 1971 *9* 64—81
LANGLEY L L and J A WHITESIDE Mechanism of accomodation and tone of urinary bladder *J Neurophysiol* 1951 *14* 147—152
LLOYD-DAVIES R. W A E CLARK W G PROUT K E D SHUTTLEWORTH and J R TICHE The effects of stretching the rabbit bladder Preliminary observations *Invest Urol* 1970 *8* 145—152
NESBIT R M and J LAPIDES Bladder tonus in spinal shock *J Urol* (Baltimore) 1948 *59* 726—732
OSBORNE W A The elasticity of rubber balloons and hollow viscera *Proc roy Soc B* 1909 *81* 485—499
PLUM F Autonomous urinary bladder activity in normal man *Arch Neurol* (Chic) 1960 *2* 497—503
SUNDBLAD R Urinary bladder dynamics in women *Scand J Urol Nephrol* 1971 *Suppl 6* 5—51
SUSSET J G p 225 in Hinman F (Ed) *Hydrodynamics of micturition* 1971 Charles C Thomas Publisher Springfield Illinois USA
TANG P C and T C RUCH Non neurogenic basis of bladder tonus *Amer J Physiol* 1955 *181* 249—257

Acta physiol scand 1974 92 496—507

From the University Institute for Experimental Medicine and the Institute for Experimental Hormone Research Copenhagen Denmark

Inhibitory Effect of Prostaglandins on Isosmotic Fluid Transport by Rabbit Gall-Bladder In Vitro, and its Modification by Blockade of Endogenous PGE-Biosynthesis with Indomethacin

By

P P Leyssac K Bukhave and O Frederiksen

Received 30 April 1974

Abstract

Leyssac P P K Bukhave and O Frederiksen *Inhibitory effect of prostaglandins on isosmotic fluid transport by rabbit gallbladder in vitro and its modification by blockade of endogenous PGE biosynthesis with indomethacin* Acta physiol scand 1974 *92* 496—507

In the presence of indomethacin serosal application of PGE_1 and $PGF_{2\alpha}$ caused significant inhibition of net isosmotic fluid transport by the gall bladder with maximum effect (50 inhibition) at 0 5 μg PGE/ml Mucosal application was much less effective $PGF_{2\alpha}$ was about 100 times less potent than PGE In the absence of indomethacin the sensitivity to low concentrations of PGE_1 was about 10 times less than in the presence of this drug ($p < 0.001$) Maximum effective doses of PGE_1 depressed the unidirectional Na efflux about 35 and the Na influx about 30 Transepithelial potential difference and ohmic resistance remained unchanged It is concluded that PGE inhibits isosmotic fluid transport by an effect mainly on the active component of the transfer process In the absence of indomethacin endogenous $PGF_{2\alpha}$ (but not PGE_1) was released to both serosal and mucosal medium Net release to mucosal medium was 35—600 pg h^{-1} mg dry weight^{-1} release was higher to the serosal medium Mucosal concentrations of 0 2—4 5 ng PGE_2/ml were independent of the serosal concentration which did not exceed 0 8 ng/ml under the present conditions The data suggest that mucosal PGE_2-release originates in part at least from the epithelial cell Indomethacin (10 μg/ml blocked almost completely the *in vitro* PGE-biosynthesis

Prostaglandins have not yet been isolated from renal cortex *However* in a recent study Larsson and Änggard (1973) demonstrated that although *in vitro* PG-biosynthesis is much higher in rabbit renal medulla than in cortex cortical tissue is capable of synthesizing appreciable amounts of PGE This had previously escaped recognition because the cortex quickly metabolizes and inactivates PGE through its high 15-hydroxyprostaglandin dehydrogenase activity (PGDH) The presence in renal lymph of PGE like material which increased in amounts after infusion of noradrenaline or angiotensin II (Fujimoto and Lockett 1970) as it does in renal

venous blood (McGiff *et al* 1970 1972) strongly suggests *in vivo* PGE biosynthesis in the renal cortex *In vivo* cortical PGE synthesis of potential physiological significance is further supported by the demonstration that renal vasoconstrictor effects of noradrenaline and angiotensin II are augmented by blockade of endogenous PGE-synthesis by indomethacin (Aiken and Vane 1973) and also by the finding that renal blood flow is reduced by indomethacin or meclofenemate in doses which block PGE release to renal venous blood (Lonigro *et al* 1973)

In view of these findings it would be of interest to know whether or not PGE affects the isosmotic fluid transfer process characteristic of renal proximal tubules The rabbit gall bladder *in vitro* preparation has transport characteristics very similar to those of renal proximal tubules (Diamond 1964) and it has been argued that with respect to fluid transport the gallbladder may serve as a suitable model system for the proximal tubular segment (Frederiksen and Leyssac 1969) The simplicity of this preparation has the advantage of avoiding not only the vascular effects but also the artefacts inherent in micropuncture methodology for direct test of nephron function (*cf* Gottschalk and Lassiter 1973)

Using the rabbit gall bladder preparation the present investigation demonstrates that PG of the E series are potent inhibitors of isosmotic net fluid transport and that the effect is due mainly to inhibition of the active component of the transfer process

Methods

Gall bladders of female white rabbits weighing 2 5—3 0 kg were removed and prepared for gravimetric measurement of net fluid transport rate as previously described (Diamond 1964 Frederiksen and Leyssac 1969) Ringer's solution was (in mEq/l) Na 114 7 K 7 0 Ca 4 0 Mg 2 4 Cl 107 0 HCO_3 17 5 SO_4 2 4 H_2PO_4 1 2 monoglutamate 5 0 glucose 11 0 mM pH was adjusted to 7 3—7 4 by equilibration with 96 % O and 4 % CO_2 at 37 C Indomethacin (10 µg/ml) was included in the media both inside and outside the bladder in all experiments with the exception of one PGE_1 series (indicated in the text) and for measurements of *in vitro* PGE biosynthesis

All experiments were carried out at 37 C and the bladders were bathed both inside and outside with the same Ringer solution The inside solution was renewed every 15 minutes The gas was continuously bubbled through the serosal medium ensuring adequate oxygenation and mixing during the experiments PGE_1 PGE or PGF a * were applied to either the serosal (outside or S) or the mucosal (inside or M) medium in final concentrations of 0 001—5 0 µg/ml in the manner described below

Measurements of fluid transport

When a steady transport rate had been reached usually 90 min after sacrifice the bladder was transferred to a second beaker containing PG (serosal application) or it was emptied and then refilled with PG-containing Ringer (mucosal application) and measurements were continued until a new steady transport rate was obtained (usually 90 min after application) The bladders were finally returned to control solution (bilaterally) for a recovery period In this way each bladder served as its own control At the end of the experiment the bladder was cut open blotted with filter paper and weighed The bladder tissue was then desiccated for 24 h at 100 C and the dry weight measured

The changes in net transport rate which are presented are corrected for the spontaneous decrease in transport rate with time observed in 12 control bladders bathed in indomethacin containing Ringer For the PGE_1 series in which indomethacin was omitted the correction

* Prostaglandins of the E and F series were generously supplied from Dr John E P Upjohn Company Kalamazoo Mich USA

was obtained from the spontaneous decrease in transport rate observed in 29 control bladders bathed in indomethacin free medium (Fig 1) Results are expressed as per cent of control transfer rate and are given as mean ± S E

Flux measurements

After steady absorption rate had been obtained the bladder was emptied refilled with Ringer solution containing 2—4 μCi ^{22}Na/ml and weighed The outside of the bladder was rapidly flushed with non radioactive Ringer and immediately transferred to a beaker containing 25 0 ml of the control Ringer 100 μl samples of the outside solution were obtained every 30 s during the flux period (6—8 *min*) for counting of radioactivity At the end of the flux measurement the bladder was weighed and emptied The specific activities both of the ^{22}Na labelled Ringer solution originally installed in the bladder and of that re aspirated at the end of the *period* were determined The bladder was then washed and refilled with non radioactive Ringer solution and transferred to the control Ringer Net transfer rate was measured for a 10—30 *min* period in order to ascertain that a significant change had not occurred The bladder was then placed in a solution containing PGE_1 (0 1—0 5 μg/ml) and net fluid transfer measurements were continued 15—30 min later flux measurements were repeated with PGE_1 at the same concentration in the serosal medium The bladder was then refilled with non labelled Ringer and net fluid transfer measured in the continued presence of PGE_1 until a steady transfer rate had been reached (60—120 min but usually 90 min from initial exposure to PGE_1) Flux measurements were then repeated and the bladder was finally transferred to the control Ringer solution for a recovery period in which net fluid transfer was *measured*

The samples were counted in a γ well scintillation counter to at least 10 000 counts or for *1000* s and corrected for background

Since within the period of measurement the decrease in specific activity was approximately a first order process the mean specific activity of the luminal solution was estimated graphically from a semi log plot of cps versus time Since the specific activity of the luminal solution decreased less than 20 % during the period of flux measurement and since the ratio of mucosal and serosal volume was at most 1/25 the specific activity of the serosal medium (and thus the Na influx) may be disregarded

The efflux rate of Na (mucosal to serosal) was obtained by plotting the radioactivity (cps) of samples of serosal medium versus time The curve became rectilinear within 3—4 min indicating a steady state unidirectional flux The efflux rate was taken as the slope of the last linear part of the curve Unidirectional sodium efflux (J_{M-S}^{Na}) across the total bladder wall (μEq/h) including active transport and exchange diffusion was calculated from the efflux of activity (J_{M-S}^{*Na}) the mean specific activity (c^*/c) and the mean volume of the serosal bathing medium ($\bar{V}_s$) according to the expression

$$J_{M-S}^{Na} = \frac{(J_{M-S}^{*Na} \cdot \bar{V}_s)}{c^*/c} \ \mu Eq/h$$

Net sodium flux (active transport) was calculated from the net fluid transfer rate and the sodium concentration of the absorbate (125 mEq/l) assuming fluid absorption isosmotic with the luminal solution and a potassium concentration slightly below 7 0 mEq/l (Diamond 1964) Net flux is defined as $J_{net} = J_{M-S} - J_{S-M}$ Unidirectional sodium influx (S—M) was calculated as the difference between unidirectional sodium efflux (M—S) and net sodium flux

Measurement of electrical potential and ohmic resistance

Gall bladders were cut open and mounted as sheets between two half-chambers with an exposed area of 0 9 cm² (Ussing and Zerahn 1951) Mucosal and serosal solutions were identical both in volume (10 0 cm³) and ion composition and were circulated by streams of humidified gas mixture (96 % O_2 and 4 % CO_2) Temperature was kept at 37 °C by means of water jackets PGE_2 was added to the serosal medium in micro-liter volumes to give a final concentration of 0 1 μg/ml

Transepithelial electrical potential difference (P D) was measured with two calomel electrodes connected to the mucosal and serosal sides via Ringer Agar bridges (1 5 mm internal diameter) placed close to the surfaces of the tissue P D was measured to the nearest 0 05 mV with a millivoltmeter The ohmic resistance (R) of the tissue was estimated from the change in transepithelial P D during passage of a brief current pulse of 100 μA through Ringer Agar bridges (2 0 mm internal diameter) placed 25 mm apart from the bladder surfaces These bridges were connected through Ag AgCl electrodes to a current source and the applied current was measured by a microammeter

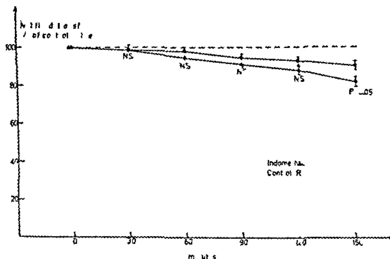


Fig 1 Spontaneous changes in net fluid transfer rate with time in gall bladders incubated with and without indomethacin (10 $\mu g/ml$) The control value (100 % time zero) was taken as the steady transport rate reached within 90 min of sacrifice Values given as m an bars indicate ± S E ● data obtained from 1. bladders incubated in Ringer solution containing indom thacin ▲ data from 29 bladders incubated in indomethacin free Ringer solution

Since the electrical resistance of rabbit gall bladder is low and of the same order of magnitude as that of the remaining consti uents of the cir uit measured values of P D and R were corrected for values recorded before mounting the gall bladder bet een the chambers R is given in terms of ohm cm

PG release

The procedure for estimation of PGE relea e from the bladder was e sentially the same as that for measurement of net fluid transfer the bladder was emptied and refilled with fr sh Ring r solution every 15 min When a steady transport rate had been reached the bladder was trans ferred to a beaker containing 25 0 ml of Ringer solution (time zero) The volume of the re aspirated luminal solution was measured nd PGE content determined in pooled 1 min samples from the first 30 m n period and the following two 60-min periods. Corresponding 1 0 ml samples of the serosal (outside) medium were coll cted at 30 90 and 150 min for measurements of PGE content

From bladders incubated in indomethacin-containing media PGE content was measured in the re aspirated inside solution pooled from the entire 150 min period and in the total outside solution at 150 min

PGE_1 and PGE_2 were measured by the isotope deri ative method d scribed by Bojesen and Buktave (1972)

Results

Effect on iso motic net fluid transport

Net fluid absorption rates by non treated gall bladders slowly decayed spontaneously with time during the experimental period as hown in Fig 1 This decrea e was slightly less in bladders incubated in Ringer solution containing indomethacin Ho ever the difference in transport capacity between the two series of bladders

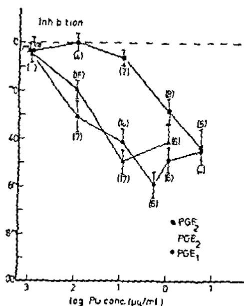


Fig 2

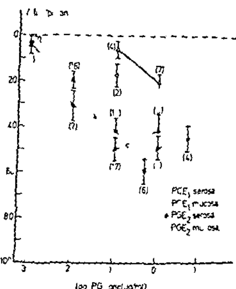


Fig 3

Fig 2 Comparison of the effect of different doses of PGE₁ PGE₂ and PGF₂α on net fluid transfer rate Inhibition in per cent of control value (ordinate) is plotted against log PGE concentration (abscissa) ● PGE₁ ▲ PGE₂ ■ PGF₂α Mean values bars indicate ± S E Numbers within brackets indicate number of experiments

Fig 3 Comparison of the inhibitory effect on net fluid transfer rate of different doses of PGE₁ and PGE₂ after serosal as opposed to mucosal application Mean values bars indicate ± S E Number of experiments within brackets

becomes significant 150 min after a steady control level had been reached (time zero)

The log dose response curves obtained after serosal application of PGE₁ F and F₂α (in indomethacin-containing Ringer) are given in Fig 2 The experimental data were obtained 90 min after application of PG i e when a steady transport rate was reached It is seen that all 3 prostaglandins inhibited net fluid transport rate The inhibitory effect of PGE_1 and PGE was quantitatively similar the small difference being insignificant At a PGE concentration of 0 01 µg/ml the effect was highly significant (about 25 % inhibition $p < 0\ 001$) at 0 001 µg/ml it was insignificant ($p > 0\ 2$) The maximum effect of around 50 % inhibition was obtained with PGE_1 and E_2 at a serosal concentration of about 0 5 µg/ml at the higher dose of 5 0 µg/ml the effect was less marked than with 0 5 µg/ml

$PGF_{2\alpha}$ was about 100 times less potent than prostaglandins of the E series

The inhibitory effect was partly reversible for all three prostaglandins tested

When PGE_1 or PGE_2 were added to the mucosal side of the epithelium net transport inhibition was much less marked than after serosal application A cor

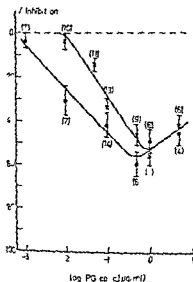


Fig 4

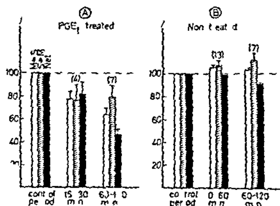


Fig 5

Fig 4 Comparison of the inhibitory effect of different doses of PGE_1 on net fluid transfer rate by gall bladders incubated with (●) and without (×) indomethacin (10 μg/ml) Mean values bars indicate ±S E Number of experiments within brackets

Fig 5 Comparison of percentage changes in unidirectional fluxes of Na with time in 4 bladders exposed to 0 1—0 5 g PGE_1/ml in the serosal medium (treated A) and in control bladders incubated without PGE_1 (non treated B) Mean values given by columns bars indicate ±S E Number of flux measurements within brackets

centration about two orders of magnitude higher was required in the mucosal than in the serosal medium to inhibit net transfer by 20 % (Fig 3)

Incubation of the bladders in Ringer solution containing indomethacin (10 μg/ml) increased the sensitivity to low concentrations of PGE_1 about 10-fold (Fig 4) At a PGE_1 concentration of 0 01 μg/ml an inhibition of 31±6 % of control value was observed with indomethacin in the medium while no significant effect was apparent at this dose in the absence of indomethacin This difference is highly significant ($p < 0\,001$) At high PGE-concentrations of 1 0 μg/ml or more in the medium no difference in response to PGE was observed between bladders incubated with and without indomethacin

Unidirectional fluxes of Na

The effects of PGE_1 applied to the serosal bathing medium at maximum effective doses (0 1—0 5 μg/ml) on the unidirectional fluxes of sodium are presented in Fig 5 A Fluxes measured at the same time intervals in control bladders not treated with PGE_1 are shown in Fig 5 B The data (Fig 5 A) show that the unidirecti-

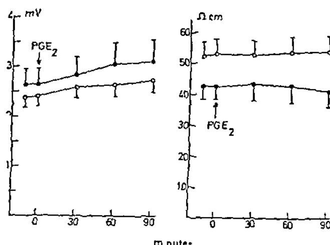


Fig 6 Comparison of changes in transepithelial PD (left) and ohmic resistance (right) with time in bladders incubated with (● N = 4) and without (O N = 14) addition of 0 1 μg PGE_2/ml to the serosal side Mean values bars indicate ± S E

Na -efflux from mucosal to serosal side (including both active transport and exchange diffusion) as well as the Na influx from serosal to mucosal side were depressed almost equally (about 20 %) within the first half hour after application of the hormone When steady net transfer rate had been reached (after about 90 min) Na efflux (M—S) had decreased about 35 % while influx (S—M) had not changed any further Thus net flux of sodium was inhibited mainly due to inhibition of the active component of the unidirectional efflux These changes in Na fluxes were absent in the parallel series of non treated bladders in which the unidirectional fluxes of sodium rather showed an insignificant tendency to increase (Fig 5 B)

Potential difference and resistance

PGE_2 at serosal concentration of 0 1 μg/ml i e at a concentration which causes maximum effect on fluid transport rate in gall bladders incubated with indomethacin had no significant effects either on transepithelial PD or on electrical conductivity of the bladder wall as apparent from Fig 6

In vitro release of PGE

PGE_1 could not be measured in detectable amounts in any of the samples (detection limit 100 pg) However Table I shows that PGE_2 was released in considerable amounts both to the inside and outside solution and Table II shows that this release was almost completely abolished by blockade of PGE biosynthesis with indomethacin 10 μg/ml In the absence of indomethacin (Table I) the PGE_2-concentration in the luminal solution was higher than that of the outside solution except

TABLE I *In vitro* release of PGE$_2$ from 3 gall bladders incubated in control Ringer solution without indomethacin

	Bladder I			Bladder II			Bladder III		
Without indomethacin	PGE$_2$ 10^{-} g/ml	Vol ml	Release 10^{-} g/h/ mgDW	PGE$_2$ 10^{-} g/ml	Vol ml	Release 10^{-} g/h/ mgDW	PGE$_2$ 10^{-} g/ml	Vol ml	Release 10^{-} g/h/ mgDW
min inside									
0–30	200	1.8	35.4	900	2.1	204	2940	1.3	344
30–90	5[illegible]5	4.0	105.0	950	4.1	200	3940	2.4	426
90–150	360	4.3	75.6	615	4.4	146	4450	2.5	595
outside									
30	500	25	1220	–	–	–	795	25	900
90	180	25	–	410	25	–	445	25	–
150	155	25	–	500	25	–	690	25	–

for one sample great differences in the luminal concentration were observed between individual bladders (range 0.2—44.5 ng/ml) and the luminal concentration of PGE$_2$ was independent of the outside concentration. Net release to the inside medium ranged from 35 to 600 ng PGE$_2$ per hour per mg dry weight (D.W.). The PGE$_2$-concentration in the outside solution was highest after the first 30 min period and then gradually decreased during the following two 60 min periods indicating loss of PGE from the outside solution with time. Therefore minimum net release of PGE to the outside solution could only be meaningfully estimated from the first 30 min samples which indicate a rate of relase considerably higher to the outside than to the inside solution.

TABLE II *In vitro* release of PGE$_2$ from 4 gall bladders incubated in control Ringer solution with indomethacin 10 μg/ml

Exp no	μg/ml indometh		PGE$_2$ 10^{-} g/ml	Vol ml	Release 10^{-} g/h/ mgDW
IV	10	inside	110	10	18.3
		outside	30	23	11.4
V	10	inside	90	11	14.9
		outside	10	23	3.5
VI	10	inside	30	14.5	4.6
		outside	23	23	5.5
VII	10	inside	45	6.5	6.7
		outside	15	22	7.6

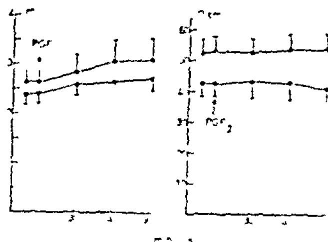


Fig. 6. Comparison of changes in transepithelial P.D. (left) and ohmic resistance (right) with time in bladders incubated with (● N = 4) and without (○ N = 4) addition of 0.1 µg PGE_2/ml to the serosal side. Mean values ± S.E.

Na-efflux from mucosal to serosal side (including both active transport and exchange diffusion) as well as the Na-influx from serosal to mucosal side were depressed almost equally (about 20%) within the first half hour after application of the hormone. When steady net transfer rate had been reached (after about 90 min) Na-efflux (M→S) had decreased about 35% while influx (S→M) had not changed any further. Thus net flux of sodium was inhibited mainly due to inhibition of the active component of the unidirectional efflux. These changes in Na fluxes were absent in the parallel series of non-treated bladders in which the unidirectional fluxes of sodium rather showed an insignificant tendency to increase (Fig. 5B).

Potential difference and resistance

PGE_2 at serosal concentration of 0.1 µg/ml, i.e. at a concentration which causes maximum effect on fluid transport rate in gall bladders incubated with indomethacin had no significant effects either on transepithelial P.D. or on electrical conductivity of the bladder wall as apparent from Fig. 6.

In vitro release of PGE

PGF_1 could not be measured in detectable amounts in any of the samples (detection limit 100 pg). However, Table I shows that PGE_2 was released in considerable amounts both to the inside and outside solution and Table II shows that this release was almost completely abolished by blockade of PGE-biosynthesis with indomethacin 10 µg/ml. In the absence of indomethacin (Table I) the PGE_2-concentration in the luminal solution was higher than that of the outside solution excep

TABLE I *In vitro* release of PGE_1 from 3 gall bladders incubated in control Ringer solution without indomethacin

	Bladder I			Bladder II			Bladder III		
Without indomethacin	PGE_1 10 g ml	Vol ml	Release 10 g h mgDW	PGE_1 10 g ml	Vol ml	Release 10 g h/ mgDW	PGE_1 10^{-1} g ml	Vol ml	Release 10 g h mgDW
min inside									
0–30	200	1 8	3 4	900	2 1	704	2940	1 3	344
30–90	5?5	4 0	105 0	950	4 1	200	3940	2 4	476
90–150	360	4 3	75	[illegible]	4 4	146	4450	2 5	595
outside									
30	500	25	1330	—	—	—	95	25	900
90	180	25		410	25	—	445	25	—
150	155	25	—	600	25	—	690	25	—

for one sample great differences in the luminal concentration were observed between individual bladders range 0 ?—445 ng/ml) and the luminal concentration of PGE was independent of the outside concentration Net release to the inside medium ranged from 35 to 600 ng PGE per hour per mg dry weight (D W) The PGE concentration in the out ide soluti n was highest after the first 30 min period and then graduall decrea ed durin the f ll wing two 60-min periods indicating loss of PGE from the out ide soluti n with time Therefore minimum net release of PGE to the out ide luti n c uld nl e meaningfully estimated from the first 30 min samples which indicate a rat f rele e considerably higher to the outside than to the inside soluti n

TABLE II [illegible] incubated in control Ringer solut n [illegible]

Exp n	[illegible]	[illegible]	PGE g ml	Vol ml	Release 10 g h mgDW
IX	1	[illegible]	110	10	
		[illegible]	30	23	18 3
X	1	d	40	31	11 4
		[illegible]	10	23	14 4
XI	1	[illegible]	30	14 5	3
		[illegible]	73	23	4
XII	1	[illegible]	45	6 5	
		ut d	15		

Discussion

Prostaglandins (PGs) have been shown to augment sodium transport and vasopressin induced water transfer across high resistance epithelia such as the frog skin and bladder and mammalian collecting duct, the effect being mediated through an interaction with membrane adenyl cyclase (Orloff, Handler and Bergstrom 1965, Grantham and Orloff 1968, Ramwell and Shaw 1970, Lipson and Sharp 1971, Omachi and Sharp 1972). These findings could have a bearing on the physiological role of endogenous prostaglandins in the renal medulla. In low resistance epithelia PG has been shown to have effects on transport which are often different from high resistance epithelia. Thus prostaglandins have also been shown to interfere with gastric acid and fluid secretion (Robert *et al.* 1967, Shaw and Ramwell 1968, Nezamis *et al.* 1971) and to inhibit intestinal sodium absorption (Al Awqati and Greenough 1972). In a recent report Matuchansky and Bernier (1973) showed that PGE_1 not only inhibited water, glucose and electrolyte net absorption from human intestine *but reversed the* net water and electrolyte movements to net secretion.

The present investigation shows that prostaglandins of the E series are potent inhibitors of isosmotic fluid transport when added to the serosal side of gall bladder epithelium. Significant inhibition was observed *in vitro* at serosal concentrations above 0.001 *μg*/ml, and the dose response curve was quantitatively similar to that given for the antilipolytic effect of PGE_1 on isolated adipose tissue of normally fed rats (Carlsson and Micheli 1970).

The gall bladder was used as an analogue of the renal proximal tubule but whether such inhibitory effects on isosmotic fluid transport are also of physiological significance in the kidney cannot be answered definitely by the present findings. The renal venous plasma concentration of PGE in normal rats is about 150—300 pg/ml (McGiff *et al.* 1972, Leyssac and Christensen, unpublished), i.e. about 3—6 times lower than the threshold concentration for the *in vitro* inhibitory effect. However the local interstitial PGE-concentration in the cortex might be higher than the renal venous plasma concentration, also the sensitivity to PGE might be higher *in vivo* than *in vitro*, finally differences in sensitivity may exist between various isosmotic transporting epithelia.

Higher renal venous plasma concentrations in the range 1—10 ng/ml have been reported after infusion of noradrenaline (McGiff *et al.* 1972 *b*), angiotensin II (McGiff *et al.* 1970, Aiken and Vane 1973), bradykinin (McGiff *et al.* 1972 a, Ferreira *et al.* 1973) and in renal hypertension (Jaffe *et al.* 1972). From the present observations such high plasma concentrations would be expected to depress isosmotic reabsorptive capacity of the proximal tubules.

The effect of i.v. infusion of PGE *in diuretic doses on* proximal tubular function has been investigated with micro-puncture technique by Fülgraff and Meiforth (1971). Proximal transit time was shortened and reabsorptive $t_{1/2}$ *increased*, indicating a fall in proximal fractional reabsorption and suggesting a decrease in absolute rate of reabsorption as well. However, they also found that end proximal tubular fluid/plasma inulin ratio measured in collected samples, was unchanged by

PGE indicating unchanged fractional reabsorption The data indicate that artefacts in one or both of the methods applied must have occurred Single nephron filtration rate was claimed to be unchanged but control data were not reported It is therefore not possible to conclude from these data whether or not PGE infusion had any effect on proximal tubular function

The present data provide some information on the mode of action of PGE on the fluid transfer process Depression of net Na absorption may occur as a result of either an increase in the passive Na backflux (influx S → M) or a decrease in the active Na efflux (M → S) or both PGE depressed unidirectional Na backflux as well as Na -efflux Inhibition of net flux thus was due to inhibition of the active component of the transfer process In low resistance epithelia such as the gall bladder the major part of the passive ion fluxes take place between the cells and across the junctional complexes which are sites of very low ohmic resistance (Fromter 1972) Any significant change in electrolyte permeability of this paracellular shunt path would be manifested in a change in ohmic resistance The data show that inhibitory doses of PGE were without any effect on either P D or resistance thereby excluding any significant permeability change of the junctional complexes The depressed backflux is therefore more likely to be due to a decreased Na permeability of the cell membrane Inhibition of the active transport could be due either to limitation of the mucosal Na and associated anion uptake or to inhibition of the pump or both Even though the data indicate a decreased Na permeability within the first half hour after addition of PGE_1 it was observed that Na efflux and net transfer continued to decrease within the subsequent hour without any further change in the backflux Thus it may be concluded that PGE depresses the active pump mechanism directly and that an additional effect on the luminal uptake of Na and its anion cannot be excluded

The gall bladder *in vitro* preparation liberated considerable amounts of PGE (about 1—1 5 ng/h/mg DW) to the bathing media and indomethacin a potent blocker of PGE biosynthesis (Vane 1971) largely abolished this release It was also shown that blockade of this endogenous PGE biosynthesis increases the sensitivity of the bladder to the transport effect of exogenous PGE about 10 times Similarly Ferreira *et al* (1972) demonstrated an increase in sensitivity to the smooth muscle effect of PGE in isolated intestine treated with indomethacin However the concentrations of endogenous PGE released to the bathing media in our experiments did not exceed 0 8 ng/ml on the serosal side or 5 0 ng/ml on the mucosal side and cannot account for the 10 fold decrease in threshold concentration (10 down to 1 ng/ml) observed in indomethacin treated bladders One explanation might be that intracellular PGE -concentration in bladders with intact PGE biosynthesis is much higher than that obtained in the bathing media and that intracellularly located PGE affects the pump Alternatively it is not excluded that indomethacin directly augments the sensitivity by an effect on the receptor or by depressing phosphodiesterase activity (Flores and Sharp 1972)

The fact that concentrations of endogenous PGE in the serosal medium did not

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FROMTER E The route of passive ion movement through the epithelium of Necturus gall bladder *J Membr Biol* 1972 *8* 259—302

FUJIMOTO S and M F LOCKETT The diuretic actions of prostaglandin E_1 and of noradrenaline and the occurrence of a prostaglandin E_1 like substance in the renal lymph of cats *J Physiol* (Lond) 1970 *208* 1—19

FULGRAFF G and A MEIFORTH Effects of prostaglandin E_2 on excretion and reabsorption of sodium and fluid in rat kidneys (Micropuncture Studies) *Pflugers Arch ges Physiol* 1971 *330* 243—256

GOTTSCHALK C W and W E LASSITER Micropuncture methodology In *Handbook of Physiology* Section 8 Renal Physiology Eds J Orloff and R Berliner The Williams and Wilkins Co Baltimore Maryland 1973 pp 129—144

GRANTHAM J J and J ORLOFF Effect of prostaglandin E_1 on the permeability response of the isolated collecting tubules to vasopressin adenosin 3 5 monophosphate and theophylline *J clin Invest* 1968 *47* 1154—1161

JAFFE B M CH W PARKER G R MARSHALL and PH NEEDLEMAN Renal concentrations of prostaglandin E in acute and chronic renal ischemia *Biochem biophys Res Commun* 1972 *49* 799—805

LARSSON C and E ÄNGGARD Regional differences in the formation and metabolism of prostaglandins in the rabbit kidney *Europ J Pharmacol* 1973 *21* 30—36

LIPSON L C and G W G SHARP Effects of prostaglandin E_1 on sodium transport and osmotic water flow in the toad bladder *Amer J Physiol* 1971 *220* 1046—1052

LONIGRO A J H D ITSKOVITZ K CROWSHAW and J C MCGIFF Dependency of renal blood flow on prostaglandin synthesis in the dog *Circulat Res* 1973 *32* 712—717

MATUCHANSKY C and J J BERNIER Effect of prostaglandin E_1 on glucose water and electrolyte absorption in the human jejunum *Gastroenterology* 1973 *64* 1111—1118

MCGIFF J C K CROWSHAW N A TERRAGNO and A J LONIGRO Renal prostaglandins possible regulators of the renal actions of pressor hormones *Nature* (Lond) 1970 *227* 1255—1257

MCGIFF J C N A TERRAGNO K U MALIK and A J LONIGRO Release of a prostaglandin E-like substance from canine kidney by bradykinin *Circulat Res* 1972 a *31* 36—43

MCGIFF J C K CROWSHAW N A TERRAGNO K U MALIK and A J LONIGRO Differential effect of noradrenaline and renal nerve stimulation on vascular resistance in the dog kidney and the release of a prostaglandin E like substance *Clin Sci* 1972 b *42* 223—233

MEZAMIS J E A ROBERT and D F STOWE Inhibition by prostaglandin E_1 of gastric secretion in the dog *J Physiol* (Lond) 1971 *218* 369—383

ORLOFF J J S HANDLER and S BERGSTROM Effect of prostaglandin (PGE_1) on the permeability response of the toad bladder to vasopressin theophylline and adenosine 3 5 monophosphate *Nature* (Lond) 1965 *205* 397—398

OZER A and G W G SHARP Effect of prostaglandins and their inhibitors on osmotic water flow in the toad bladder *Amer J Physiol* 1972 *222* 674—680

RAMWELL P W and J E SHAW Biological significance of prostaglandins In *Rec Progr Horm Res* 1970 26 139—173 Ed E B Astwood Acad Press N Y

ROBERT A J E NEZAMIS and J P PHILLIPS Inhibition of gastric secretion by prostaglandins *Amer J dig Dis* 1967 *12* 1073—1076

SHAW J E and P W RAMWELL Inhibition of gastric secretion in rats by prostaglandin E_1 In *Prostaglandin Symposium* Worcester Foundation for Exper Biol Interscience 1968 New York John Wiley & Sons pp 55—64

USSING H H and K ZERAHN Active transport of sodium as the source of electrical current in the short-circuited isolated frog skin *Acta physiol scand* 1951 *23* 110—127

VANE J R Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs *Nature New Biol* 1971 *231* 232—235

Acta physiol scand 1974 92 508—516

From The University Institute for Experimental Medicine Copenhagen Denmark

The Effect of Angiotensin on Isosmotic Fluid Absorption by the Rabbit Gall-Bladder In Vitro

By

P P Leyssac L Østergaard Kristensen, P Christensen and O Frederiksen

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Abstract

Leyssac P P I Østergaard Kristensen P Christensen and O Frederiksen *The effect of angiotensin on isosmotic fluid absorption by the rabbit gall bladder in vitro* Acta physiol scand 1974 *92* 508—516

The rabbit gall bladder *in vitro* preparation was used for studying the effect of angiotensin II on isosmotic fluid transport Angiotensin inhibited net fluid transfer at serosal concentrations between $10^{-9}-10^{-8}$ M Maximum inhibition (about 25) was obtained at a concentration of 5×10^{-8} M *No significant effect was demonstrable at concentrations of* 10^{-10} *M or lower and the effect disappeared* at higher concentrations (10 and 20×10 M) The inhibitory effect was due to a depression of the unidirectional Na^+-efflux (from mucosal to serosal side) Na^+ influx (from serosal to mucosal side) was unaffect d Maximum effective doses of angiotensin had no effect on transepithelial potential difference (PD) while ohmic resistance (R) increased slightly (about 10 %) The inhibitory effect of angiotensin was significantly reduced by incubating gall bladders with indomethacin (10 μg/ml) which effectively blocks prostaglandin biosynthesis Addition of indomethacin alone had no effect on either net fluid transfer rate PD or R It is concluded that angiotensin inhibits isosmotic fluid transport in the gall bladder by an effect on the active component *of the transfer process and the data suggest that the effect is indirect and mediated in part at least* by release of endogenous prostaglandins Alternatively indomethacin might interfere with the angiotensin receptor

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The location of the renin angiotensin system in the juxtaglomerular apparatus and the renal effects of administered angiotensin II have raised the possibility of a local physiological pathophysiological action of angiotensin in proximal and/or distal *tubular segments of the nephron* (Leyssac 1966)

Because of the structural and functional complexity of the kidney and technical difficulties inherent in micropuncture methodology (Gottschalk and Lassiter 1973) a variety of other epithelial tissues have been studied as model systems for the various renal tubular segments Evidence has accumulated that angiotensin *can affect* transepithelial fluid and/or sodium transport in such epithelia *in vitro* but the findings are conflicting and depend apparently on the type of epithelium studied and the experimental conditions Thus while Barbour *et al* (1962) were unable to detect an effect of angiotensin on sodium transport by the toad skin stimulation was reported by McAfee and Locke (1967) using frog skin Low *concentrations of angio*tensin II ($10^{-10}-10^{-12}$ M) increased net fluid transfer by isolated everted sacs *of rat*

jejunum ileum and intact colon prepared from adrenalectomized nephrectomized rats while higher concentrations (10^{-10}–10^{-12} M) caused inhibition of net fluid transfer across stripped colonic epithelium of such rats (Davies *et al* 1970) These dual effects were confirmed by Hornych *et al* (1973) who also demonstrated that at all concentrations angiotensin stimulated sodium and water transfer in ascending but inhibited sodium and water transfer in descending colon of normal rats

Transport characteristics of the rabbit gall bladder epithelium are very similar to those of proximal tubules (Diamond 1964) and it has been argued that with respect to isosmotic fluid transport this epithelium may serve as a suitable model for the proximal tubule (Frederiksen and Leyssac 1969) In a previous study we presented preliminary evidence that angiotensin inhibited net fluid transport by the gall bladder when applied to the serosal side (Frederiksen and Leyssac 1969) In the present study this observation is examined in further detail

Methods

Gall bladders of female white rabbits weighing 2 5–3 0 kg were removed and prepared for gravimetric measurement of net fluid transport rate as previously described (Diamond 1964 Frederiksen and Leyssac 1969) Ringer solution was (in mEq/l) Na^+ 114 7 K 7 0 Ca 4 0 Mg 2 4 Cl 102 0 HCO_3^- 17 5 SO 2 4 HPO 1 2 monoglutamate 5 0 mM glucose 11 0 mM pH was adjusted to 7 3–7 4 by equilibration with 96 O and 4 CO at 37 C In 10 control and 9 angiotensin expts the Ringer solution contained indomethacin 10 μg/ml both inside and outside

All experiments were carried out at 37 C and the bladders were bathed both inside and outside with the same Ringer solution. The inside (mucosal or M) solution was renewed every 15 min The gas was continuously bubbled through the serosal (outside or S) medium ensuring adequate oxygenation and mixing during the experiments In experimental periods synthetic angiotensin II (val 4 angiotensin II amide (CIBA)) was added to the serosal medium to give the desired final concentration of 10^{-8}–$2 \times 10^{-?}$ M All glassware was siliconized to prevent loss of angiotensin by adsorption

Measurement of net fluid transport

Net fluid transfer rate was measured during a control period and when a steady transport rate had been reached the bladder was transferred to a second beaker containing angiotensin (serosal application) and measurements were continued until a new steady transport rate was obtained (usually within 60–90 min after application) In this way each bladder served as its own control At the end of the experiment the bladder was cut open blotted on filter paper and weighed The tissue was then desiccated for 24 h at 100 C and the dry weight measured

The presented changes in net transport rate are corrected for the spontaneous decrease in transport with time observed in 29 bladders incubated in indomethacin free Ringer and in 12 bladders incubated in Ringer solution containing indomethacin (10 μg/ml) respectively Results are expressed as per cent of control transfer rate and given as mean ± S E

Measurement of mucosal-to-serosal flux

Flux measurements were performed as described in detail in the preceding paper (Leyssac *et al* 1974) After a steady transport rate had been obtained the bladder was emptied and then refilled with Ringer solution containing 2–4 μC [illegible] /ml and weighed The outside of the bladder was rapidly flushed with non radioactive Ringer and immediately transferred to a beaker containing 25 0 ml of the control Ringer 100 μl samples of the outside solution were obtained every 30 s during the flux period (1–8 min) for counting of radioactivity At the end of the flux measurement period the bladder was weighed and emptied The specific activities both of the labelled Ringer solution originally instilled in the bladder and of that separated at the end of the period were determined After washing with non radioactive Ringer new transfer measurements were continued in order to ascertain that a significant change had not occurred The bladder was then transferred to a beaker with Ringer solution containing angiotensin 4 4 10 M and when a new steady transport rate was obtained (within 60–90 min) flux measurements were repeated with angiot[illegible] at the same concentration in the serosal medium

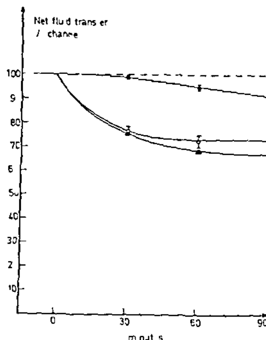


Fig 1

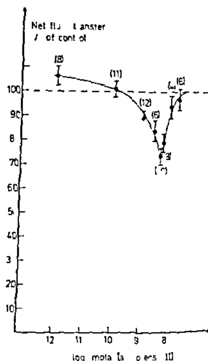


Fig 2

Fig 1 Percentage changes in net fluid transfer with time with and without 4 4 × 10 M ang oten sin in the serosal medium ● data from control bladders (spontaneous changes) ▲ data from bladders exposed to angiotensin ○ data from bladders exposed to angiotensin corrected for spontaneous changes Bars indicate ± S E

Fig 2 Log dose response of angiotensin on net fluid transfer rate

The samples were counted in a γ well scintillation counter to at least 10 000 counts for for 1000 s and corrected for background

The efflux rate of ²²Na (M—S) was obtained by plotting cps in samples of serosal m dium against t me and is given by the slope of the last linear part of the curve indicating steady state of unidirectional flux (cf Fig 3) Unidirectional sod um efflux(J^{Na}_{MS}) across the total bladder wall (μEq/h) was calculated from the efflux of acti ity $\left(J^{*Na}_{MS}\right)$ the mean specif c act vity (c c) and the mean volume of the serosal bath ng medium ($\bar{V}^s$) according to the expression

$$J^{Na}_{MS} = \frac{\left(J^{*Na}_{MS}\ \bar{V}_s\right)}{c\ /c}\ (\mu\ \mathrm{Eq/h})$$

Net sodium flux was calculated from the net fluid transport rate and the od um concentration of the absorbate (125 mEq/l) assuming fluid absorption isosmotic with the luminal solution and a potassium concentration slightly below 7 0 mEq/l (Diamond 1964) $J_{net} = J_{M-S} - J_{S-M}$

Unidirectional sodi im influx (S—M) was calculated as the difference bet een unid rectional Na-efflux and net Na flux

Measu ement of el potential difference (P D) and ohm c resistance (R)
Transepithelial el P D and R were measured as described in deta l in the preced ng paper (Leyssac et al 1974) in gall bladders mounted as sheets between two half-chambers of the Uss ng type The exposed area of epithel um was 0 90 cm and the volumes of both serosal and mucosal media were 10 0 cm Angiotensin II was added in microliter olumes to the serosal side to give final concentrations or 5 or 20 × 10 M

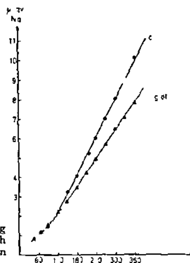


Fig 3 Data from a representative experiment showing cumulative total unidirectional Na efflux (M—S) with time in the control period (●) and after serosal addition of 4 4 × 10⁻ M angiotensin (▲) to the same bladder

Results

Fig 1 shows the percentage change in net fluid absorption rate with time in 29 control bladders and in 10 bladders exposed to angiotensin $4\ 4 \times 10^{-8}$ M in the serosal medium Time zero is taken from the time of hormonal application *i e* from the end of the control period when transport rate had remained stable for about 30 min Net transport declined gradually with time in control experiments such that at 90 min transfer rate had decreased 9 % below the initial level With $4\ 4 \times 10^{-8}$ M angiotensin in the serosal medium the decline was augmented This inhibition of net absorption rate was significant 30 min after exposure to the peptide ($p < 0\ 005$) and maximum inhibition was reached within 60 min (paired *t* test analysis)

The effect of various doses of angiotensin on net fluid transfer is given in Fig 2 At the lowest concentration tested (10^{-12} M) a slight but statistically insignificant transport stimulation was observed (6 ± 4 %) Also within the range of concentrations 10^{-11} to 10^{-8} M no significant effect could be demonstrated Only at concentrations between 10^{-8}—10^{-6} M was significant inhibition witnessed Maximum inhibition was recorded at concentrations of $4\ 4$—$5\ 5 \times 10^{-6}$ M ($p < 0\ 001$) but the effect was modest (mean 25 % inhibition) and quite variable from one preparation to another Next the effect was unrelated to the preceding control net transport rate At the highest concentrations tested (10 and 20×10^{-6} M) the inhibitory effect had virtually disappeared

The effect of angiotensin was reduced in bladders prepared and incubated in Ringer solution containing indomethacin (10 μg/ml) a potent blocker of prostaglandin biosynthesis (Vane 1971) In 9 expts (maximum effective dose 5×10^{-6} M) of angiotensin caused only $8\ 6 \pm 2\ 2$ % inhibition as compared to $23\ 9 \pm 2\ 7$ % inhibition observed in 19 bladders incubated in indomethacin free Ringer The difference is statistically significant ($p < 0\ 005$) Addition of indomethacin (10 μg/ml) alone had no effect on the rate of fluid transfer (10 expts)

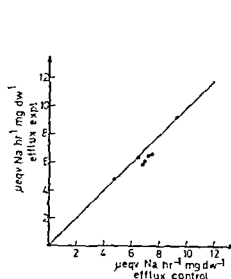


Fig 4

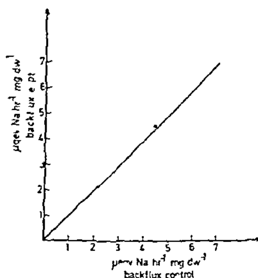


Fig 5

Fig. 4. Total unidirectional Na-efflux (M→S) in the presence of 4.4×10^{-9} M angiotensin (ordinate) against the Na-efflux measured in the preceding controol period (abscissa). The line indicates line of identity.

Fig. 5. Total unidirectional Na influx (S→M) in the presence of 4.4×10^{-9} M angiotensin (ordinate) against the Na influx measured in the preceding control period (abscissa). The line indicates line of identity.

Unidirectional Na fluxes

Data from a typical experiment are shown in Fig. 3. The total amounts of Na transferred from the mucosal into the serosal medium (ordinate) were calculated from the measured ²²Na activity accumulating in the serosal medium and the mean specific activity of the mucosal fluid. When plotted against time (abscissa) the unidirectional Na-efflux is given from the slope of the last linear part of the curve. A shift to the right from the control Na-efflux line indicates depressed efflux.

The results from all 10 expts. are presented in Fig. 4 and 5. The unidirectional fluxes after exposure to angiotensin at a concentration of 4.4×10^{-9} in the serosal medium is plotted as a function of the corresponding fluxes measured in the preceding control period. The data indicate that angiotensin depressed the mucosal to serosal efflux of sodium in 7 of 10 expts. (Fig. 4). The decrease was statistically significant ($p < 0.001$). In contrast the serosal to mucosal influx (backflux) was unaffected by angiotensin which is shown in Fig. 5 as values distributed around the line of identity. Thus the flux ratio $\left(\frac{J_{M\text{-}S}}{J_{S\text{-}M}}\right)$ was reduced by angiotensin from a mean value of 2.01 in control periods to 1.60 after addition of angiotensin.

Electrical potential difference (PD) and ohmic resistance (R)

Table I and II summarize the results of measurements of *PD* and R, respectively. It appears that in control medium (without angiotensin) transepithelial PD was

TABLE I Effect of serosal Angiotensin II on transepithelial potential difference (mV)[1]

Medium	Control period	Change in potential difference after[2]		
		30 min	60 min	90 min
Control Ringer (n = 14)	2 40±0 20	+0 18±0 08 p 0 05	+0 26±0 12 N S	+0 35±0 14 p 0 05
Angiotensin II 5×10 M (n = 4)	2 78±0 34	+0 01±0 15 N S	+0 23±0 20 N S	+0 28±0 27 N S
Angiotensin II 20×10 M (n = 4)	2 49±0 61	+0 30±0 18 N S	+0 47±0 16 N S	+0 45±0 15 N S
Angiotensin II 5×10 M (n = 4)	3 0?±0 42	+0 20±0 04 p 0 025	+0 27±0 08 p 0 05	—

[1] Mucosal side positive
[2] Time after application of angiotensin
[3] S E

increasing slightly with time the change being on the border line of significance ($p < 0.05$ Table I) Ohmic resistance remained unchanged (Table II) In two additional experiments it was found that indomethacin was without effect on these parameters The presence of angiotensin in the serosal medium at concentrations which gave maximum inhibition of net fluid transport (5×10^{-8} M or higher) had no significant effect on the PD within 90 minutes (Table I) nor did angiotensin affect PD in bladders incubated with indomethacin containing Ringer However it

TABLE II Effect of serosal Angiotensin II on epithelial resistance (Ω cm)

Medium	Control period	Change in epithelial resistance after		
		30 min	60 min	90 min
Control Ringer (n = 14)	53 0±4 9	−0 21±0 95 N S	+0 77±1 75 N S	−0 91±1 95 N S
Angiotensin II 5×10 M (n = 4)	34 2±5 3	+1 35±0 55 N S	+3 08±0 43 p 0 01	+3 94±0 [illegible] p 0 005
Angiotensin II 20×10 M (n = 4)	45 5±6 0	−0 23±1 65 N S	−0 23±2 98 N S	−0 56±3 65 N S
Angiotensin II 5×10 M	33 1±6 3	+0 68±0 83 N S	−2 14±1 12 N S	—

[1] Time after application of angiotensin
[2] S E

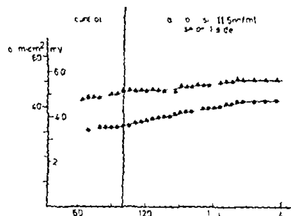


Fig 6 Transepithelial potential difference (●) and ohmic resistance (▲) before and after serosal addition of 5 × 10 M angiotensin Data from a representative experiment

appears from Table II that 5 × 10⁻⁹ M of angiotensin caused transepithelial resistance to increase slightly only about 10 % and the change was significant within 60 min this small effect was reduced by the presence of indomethacin and abolished at the high angiotensin concentration of 20 × 10⁻⁹ M Data from a representative experiment are shown in Fig 6

Discussion

The present data support the previous finding that angiotensin inhibits isosmotic water and electrolyte transport by rabbit gall bladder *in vitro* (Frederiksen and Leyssac 1969) Inhibition of net Na transport may be due either to increased passive backflux or to inhibition of the active pump mechanism The present results indicate that inhibition is due to a reduction in the unidirectional Na efflux from mucosal to serosal medium i e a reduction in the active component of the transcellular transport mechanism leaving backflux (influx) unchanged The low resistance of this type of epithelia is due to the high ionic conductance of the junctional complexes as compared to that of the cell membranes (Fromter 1972) the junctional complexes therefore constitute a paracellular shuntpath through which a significant part of the passive fluxes probably take place Any significant change in conductance of the shunt path should therefore manifest itself by a considerable change in ohmic resistance as well as in the backflux The present observation that ohmic resistance of the bladder wall did not change by more than 10 % after addition of angiotensin is therefore consistent with the flux data

In the preceding paper (Leyssac *et al* 1974) it was demonstrated that the *in vitro* gall bladder preparation synthesizes and releases considerable amounts of PGE_1 to the bathing media and that this release was blocked by indomethacin Furthermore it was shown that prostaglandins (PG) of the E series are potent inhibitors of isosmotic fluid transport by the gall bladder In the present study it was observed that the inhibitory effect of angiotensin on fluid transport was significantly reduced in bladders

incubated with indomethacin Indomethacin has also been shown to inhibit selectively angiotensin induced contractions of isolated smooth muscle preparations from ileum, fundus and aorta of rats and guinea pigs (Chong and Downing 1973) These findings suggest that indomethacin might block the angiotensin receptor or alternatively that the effects of angiotensin on gall bladder fluid transport and smooth muscle contraction are indirect and mediated in part at least, by prostaglandin liberation This latter alternative exposes the possibility that the inhibitory effect of exogenous angiotensin on renal proximal tubular reabsorption *in vivo* might also be explained by PGE-release If this were the case it follows that endogenous release of angiotensin intrarenally might exert a physiological regulatory role on proximal reabsorption rate through such a mechanism

In previous work an inhibitory effect of angiotensin II on renal proximal tubular reabsorption rate was found *in vivo* only at high rates of net transport in rats anaesthetized with the oxybarbiturate sodium Amytal (Leyssac 1964 1965) Such an effect of administered angiotensin could not be reproduced using the split oil drop method in rats anaesthetized with the thiobarbiturate Inactin (Horster *et al* 1966 Lowitz *et al* 1969) whereas a significant inhibition of distal sodium transport was demonstrated These contrary results may be partly explained by the finding that proximal—but not distal rates of reabsorption are inhibited by anaesthetic levels of Inactin but not Amytal (Elmer *et al* 1972) and partly by the limitations of the split drop method (cf Gottschalk and Lassiter 1973) In microperfusion studies of isolated segments of rabbit proximal tubules *in vitro* Burg and Orloff (1968) were also unable to find a significant effect of angiotensin (2.5×10^{-6} M) on net isometic fluid transfer In their experiments however net fluid transfer rates were less *in vitro* than *in vivo* and the dose applied was extremely large either of which factors might have concealed a potentially physiological effect

Evidence has been reported suggesting that the transport effects of angiotensin on colonic epithelium is mediated by cyclic AMP (Hornych *et al* 1973) Many of the effects of prostaglandins including effects on transporting epithelia are manifest in those systems where cycl AMP is mediating the response of the hormone and PG affects the adenyl cyclase in those systems (for review see Ramwell and Shaw 1970) Accordingly Pierce *et al* (1971) obtained evidence that the inhibitory effect of PG on jejunal net water and electrolyte absorption was greatly augmented by theophylline which elevates the cyclic AMP level intracellularly by inhibiting the phosphodiesterase which converts cyclic AMP to AMP (Butcher and Sutherland 1962) Considering the inhibitory effects of PGE and angiotensin on isosmotic fluid transport by the gall bladder and the possible interrelationship between these two effects it seems likely that cyclic AMP is also involved in the isosmotic fluid transfer mechanism and its regulation

The use of the gall bladder as an analogue of the proximal tubule has demonstrated the need to re examine renal isosmotic transport under more favourable conditions If the analogy is true it is likely that local renal hormones do function in a [illegible] manner on proximal tubular transport under physiological conditions

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References

BARBOUR B H J R GILL JR and F C BARTTER The effect of angiotensin II on sodium transport in the toad skin *Clin Res* 1962 *10* 92

BURG M B and J ORLOFF Control of fluid absorption in renal proximal tubule J *Clin Invest* 1968 *47* 2016—2024

BUTCHER R W and F W SUTHERLAND Adenosine 3 5 mono-phosphate in biological materials I Purification and properties of cyclic 3 5 nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3 5 phosphate in human urine J *biol Chem* 1962 *237* 1244—1250

CHONG E K S and O A DOWNING Selective inhibition of angiotensin induced contractions of smooth muscle by indomethacin J *Pharm Pharmacol* 1973 *25* 170—171

DAVIES N T K A MUNDAY and B J PARSONS The effect of angiotensin on rat intestinal fluid transfer J *Endocr* 1970 *48* 39—46

DIAMOND J M The mechanism of isotonic water transport J *gen Physiol* 1964 *48* 15—42

ELMER M P C ESKILDSEN L OSTERGAARD KRISTENSEN and LEYSSAC P P A comparison of renal function in rats anaesthetized with Inactin and sodium Amytal *Acta physiol scand* 1972 *86* 41—58

FREDERIKSEN O and P P LEYSSAC Transcellular transport of isomotic volumes by the rabbit gall bladder *in vitro* J *Physiol* (*Lond*) 1969 *201* 201—224

FROMTER E The route of passive ion movement through the epithelium of Necturus gall bladder J *Membr Biol* 1972 8 259—302

GOTTSCHALK C W and W E LASSITER Micropuncture methodology In *Handbook of Physiology* Section 8 Renal physiology Eds J Orloff & R Berliner The Williams & Wilkins Co Baltimore Maryland 1973 pp 129—144

HORNYCH A PH MEYER and P MILLIEZ Angiotensin vasopressin and cyclic AMP effects on sodium and water fluxes in rat colon *Amer* J *Physiol* 1973 *224* 1223—1229

HORSTER M W NAGEL J SCHNERMANN and K THURAU Zur Frage einer direkten Angiotensinwirkung auf die Natriumresorption im proximalen Tubulus und in der Henleschen Schleife der Rattenniere *Pflügers Arch ges Physiol* 1966 *292* 118—128

LEYSSAC P P The *in vivo* effect of angiotensin on the proximal tubular reabsorption of salt in rat kidneys *Acta physiol scand* 1964 *62* 436—448

LEYSSAC P P The *in vivo* effect of angiotensin and noradrenaline on the proximal tubular reabsorption of salt in mammalian kidneys *Acta physiol scand* 1965 *64* 167—175

LEYSSAC P P The regulation of proximal tubular reabsorption in the mammalian kidney *Acta physiol scand* 1966 *70* suppl 291

LEYSSAC P P K BUKHAVE and O FREDERIKSEN Inhibitory effect of prostaglandins on isosmotic fluid transport by rabbit gall bladder *in vitro* and its modification by blockade of endogenous PGE-biosynthesis with indomethacin *Acta physiol scand* 1974 *92* 496—507

LOWITZ H D K O STUMPE and B OCHWADT Micropuncture study of the action of angiotensin II on tubular sodium and water reabsorption in the rat *Nephron* 1969 *6* 173—187

MCAFEE R D and W LOCKE Effect of angiotensin amide on Na isotope flux and short circuit current of isolated frog skin *Endocrinology* 1967 *81* 1301—1305

PIERCE N F CH C CARPENTER H L ELLIOTT and W B GREENOUGH Effects of prostaglandins theophylline and cholera exotoxin upon transmucosal water and electrolyte movement in the canine jejunum *Gastroenterology* 1971 *60* 22—32

RAMWELL P W and J E SHAW Biological significance of the prostaglandins *In Re Pro Horm Res* Ed E B Astwood Acad Press New York 1970 vol 26 pp 139—173

Acta physiol. scand 1974 92 517—525

From the Department of Physiology and Medical Biophysics Biomedical Center University of Uppsala, Sweden

Increased Inulin Absorption from the Cat Stomach Exposed to Acetylsalicylic Acid

By

GUNNAR FLEMSTRÖM and N. V. B. MARSDEN

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Abstract

FLEMSTRÖM G and N. V. B. MARSDEN *Increased inulin absorption from the cat stomach exposed to acetylsalicylic acid* Acta physiol scand 1974 92 517—525

Polydisperse ^{14}C-inulin was absorbed from the stomach of the cat when 15 mM acetylsalicylic acid mainly in the unionized form in isotonic NaCl solution was also present in the lumen. The absorbed inulin was rapidly excreted and accumulated in the urine in sufficient quantities for molecular weight distribution analysis. The latter showed that about 95 percent of the urinary inulin had a molecular weight greater than 2000 and that only moderate molecular sieving of the instilled inulin (weight average molecular weight 5200) occurred. This means very probably that larger molecular species than tested here could be absorbed from the stomach. The instillation of ^{14}C-inulin in acetylsalicylic acid free isotonic NaCl, however also resulted in a smaller but significant inulin absorption. The latter was further considerably reduced if the inulin was not added together with the isotonic saline but later (20 min) in a small liquid volume. This suggests that mere distension of the stomach causes a temporary increase of the permeability which however is much smaller than that after acetylsalicylic acid. It may be relevant that acetylsalicylic acid was found here to increase gastric absorption of saccharide molecules of a size earlier reported to be sufficiently large to evoke immune reactions

It is well known that the presence of unionized acetylsalicylic acid (ASA) in the lumen of the stomach increases the ionic permeability of the gastric mucosa (Davenport 1964 1967 Frenning 1971 Ivey 1971 Smith *et al* 1971). Some other weak acids exert a similar effect although the concentrations required usually are higher (Davenport 1964 Flemström, Frenning and Öbrink 1964 Flemström and Frenning 1968 Flemström 1971). ASA is also known for its ability to evoke a diversity of allergic reactions in man (Samter and Beers 1968). In the frog gastric mucosa *in vitro* unionized ASA increased the permeability not only for ions but also larger molecules. Thus, dextran migrated from the luminal to the serosal (Flemström and Marsden 1973) the upper limit for penetration being a [illegible] weight of about 30 000. In view of the antigenicity of some polysac[illegible]

berger, Aisenberg and Hassid 1954, Kabat and Berg 1953, Richter 1972) we considered it of interest to extend this study of ASA induced absorption of macromolecules to the mammalian stomach *in vivo*. Previously salicylic acid (Davenport 1966) and sulphydryl reagents (Davenport 1971) have been reported to increase the leakage of plasma proteins into the gastric lumen.

This paper reports *in vivo* experiments on the absorption of the ^{14}C labelled polysaccharide inulin from the stomach of the cat. Inulin is a very suitable test solute in this respect. Unlike dextran it appears to be essentially inert in the body, is excreted rapidly and reaches high concentrations in the urine (Gaudino, Schwartz and Levitt 1948, Eggleton and Habib 1951). The latter is especially advantageous here since it facilitates estimation of the molecular weight distribution of the inulin which has passed through the gastric mucosa.

Materials and methods

^{14}C carboxyl labelled inulin (Radiochemical Centre, Amersham, England), referred to later as the "original fraction", had a specific activity of 1.9 μCi/mg and a weight average molecular weight ($\overline{M}_w$) of 5200. This was used without any added non radioactive inulin.

Cats weighing between 2.0 and 3.2 kg were starved for 18 h before anesthesia, but were allowed free access to water. Anesthesia was induced with chloroform; subsequently chloralose (60 mg/kg b. wt.) and urethane (~ 100 mg/kg b. wt.) were administered intravenously together with 6.0—7.5 ml isotonic NaCl solution per kg b. wt. just before the operative procedure began. During the experiment, *i.e.* when test solutes were present in the stomach, 100 mM NaCl was infused i.v. at a rate of 1 ml per kg b. wt. each hour; this resulted in a steady urine output. The body temperature was maintained at 38.5 °C ± 0.5 °C in all experiments by a heater controlled by an intrarectal thermometer.

The cats were tracheotomized as soon as sufficient depth of anesthesia was attained. The abdomen was then opened by a median incision. The stomach was isolated by ligatures round the cardia and pylorus, special care being taken to minimize interference with the blood supply. A glass canula introduced carefully through the pyloric wall into the lumen was tied in place by two ligatures, one enclosing the mucosa only, the other the whole stomach wall. A thin polyethylene tube was inserted into each ureter about 5 mm above the bladder and passed up until a urine flow was evident. It was then tied in place with a fine silk ligature.

30—50 min after completion of the above preparations the stomach was washed three times with isotonic NaCl solution.

In order to test inulin excretion in the urine 1.25 μCi inulin in 2 ml isotonic NaCl solution were injected intravenously into 3 cats and the urine flow and ^{14}C activity were monitored for 6 h. In these experiments the stomach was prepared as described above for the instillation experiments.

In the instillation experiments the solutions were always made isotonic with NaCl. At the termination of each experiment the gastric contents were retrieved and the mucosa inspected for obvious damage. These experiments were of 3 types.

(a) In the first type of experiment 6 ml of an isotonic solution containing 12.5 μCi inulin either with or without 15 mM ASA were introduced into the stomach. With ASA the pH was 2.70, *i.e.* 86 per cent was unionized. The experiment lasted 6 h and the urine volume and ^{14}C activity were measured at hourly intervals.

(b) The aim of these experiments was to compare the difference between the effects of isotonic NaCl alone and isotonic NaCl ASA (15 mM) on inulin absorption in the *same* cat. Six ml of an isotonic NaCl inulin (12.5 μCi) solution was instilled into the stomach and allowed to remain there for 3 h. It was then removed and the stomach was rinsed *three* times with isotonic NaCl ASA (15 mM). Six ml of an isotonic solution containing 12.5 μCi inulin and 15 mM ASA were then instilled and allowed in turn to remain there for 3 h.

(c) For reasons discussed below the instillation schedule in the consecutive *type* of experiment (b) was modified. The NaCl and NaCl ASA periods were each subdivided into a first inulin free and a second inulin containing part. First 5 ml of an inulin free *isotonic* NaCl solution were instilled; 20 min later 12.5 μCi inulin in 1 ml isotonic solution were added care-

fully through a soft rubber tube inserted into the glass gastric cannula One ml of the gastric contents was then withdrawn and reintroduced this procedure being repeated several times to promote mixing 3 h later the stomach was emptied and washed 3 times with isotonic NaCl ASA (15 mM) as in (b) Five ml of isotonic NaCl ASA (15 mM) were then instilled and 90 min later 1 ml of an isotonic inulin (12 5 μCi) solution also containing 15 mM ASA were added as described for the first part The urine flow and ¹⁴C activity were measured during the two 3 h periods when inulin was present in the stomach

¹⁴C activity in the urine was determined by liquid scintillation (Beckman CPM 200 or 250) after adding 0 05 or 0 10 ml urine to 10 ml scintillation fluid (Bray 1960) At least 3000 counts above background were recorded

After termination of an experiment the stomach was opened for visual inspection The gastric contents were removed and the volume pH and inulin concentration determined The presence of hemoglobin was tested for with an orthotidine reaction (Labstix Miles Ltd England) This methods was checked with standard hemoglobin solutions and the sensitivity was high giving a positive reaction with a concentration as low as 3 mg/liter

For molecular weight distribution analysis the urine samples were pooled if necessary to obtain sufficient radioactivities in the eluted samples The urine sample was first reduced to a volume of about 2 ml in a rotary vacuum evaporator The resulting solution was yellow and turbid and was clarified by pressure filtration through a 0 45 μm pore 13 mm diameter Millipore filter (HAWP 01300) the filtrate being transferred directly to the chromatographic column (gel bed volume 50 ml)

The molecular weight distribution of the ¹⁴C inulin was determined by gel chromatography on Sephadex G 25 at 4 0 ±0 1 C The linear flow rate was about 7 cm/h A high molecular weight dextran (Blue dextran $\bar{M}_w$ 2 000 000 Pharmacia Fine Chemicals Uppsala Sweden) and tritiated water were used as reference solutes (Marsden 1965) Ideally the column should be calibrated with the individual inulodextrin oligomers but as these behave approximately as the maltodextrins about which data was available (Haglund Marsden and Östling unpublished results) the latter were used for calibration instead

Since the main purpose of the gel chromatography was to exclude the presence of small molecular components in the inulin rather than to determine accurately the complete molecular weight distribution the gel type G 25 was selected This gel has upper molecular weight exclusion limits of 4000—5000 for inulodextrins which means that it cannot resolve molecular species in or above this range On the other hand it has a high resolution in the lower molecular weight range of inulin and is very suitable for detecting relatively small changes in molecular weights below about 3000

In order to test the stability of inulin in the acid conditions likely in the stomach it was exposed to different hydrogen ion concentrations (pH 1 5—7) in a 15 mM ASA buffer at 37 C for 3 h Inulin (12 μC) was mixed with 2 ml of an isotonic (NaCl) solution containing 15 mM ASA and the pH was adjusted to the required value with either 0 1 mM HCl or NaOH After 3 h readjustment to pH 7 was made and the samples were then frozen until chromatographed

Results

Treatment of the original fraction at 37° C for 3 h at different pH values did not affect the ¹⁴C elution activity profiles except at pH 1 5 At this pH there was extensive hydrolysis with a low molecular peak corresponding to fructose Fig 1 shows the elution profiles at pH 2 and pH 7 which did not differ significantly

As shown in Table I some 83 percent of the intravenously injected ¹⁴C activity was recovered in the urine within 6 h about 60 percent being excreted during the first hour The molecular weight distribution of the ¹⁴C activity excreted during the first hour was estimated The peak molecular weight was a little less than that of original fraction the shift being somewhat less than in the ¹⁴C activity excreted after intragastric instillation of inulin (see below)

The results of the consecutive 3 h instillation experiments (types b and c) are summarized in Table II and the results of two 6 h single instillation experiments

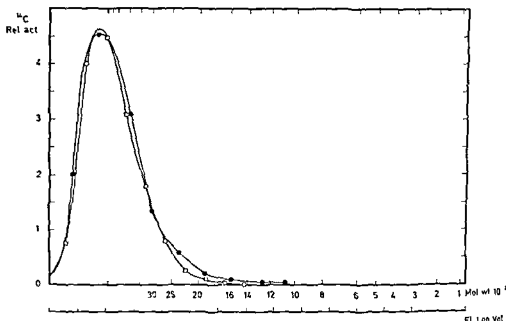


Fig 1 Effluent activity profiles of ^{14}C inulin treated for 3 h at pH 7 (open circles) or pH ? (filled circles) at 37 C after elution through a column of Sephadex G 25 The molecular weight scale was calculated from the elution behaviour of individual maltodextrin oligomers Note that the molecular weight scale becomes uncertain above about 3000

one with ASA and one without are given in Fig 2 After instillation of ^{14}C inulin and NaCl into the stomach some ^{14}C activity was found in the urine This reached a low peak value in the second h and thereafter declined slowly This peak was never more than 20 percent of the maximal value obtained after ASA treatment If ^{14}C inulin was not introduced until 20 min after instillation of the isotonic NaCl solution the ^{14}C activity in the urine was much lower The highest value obtained during the second hour was now only about 5 percent of the peak value obtained after ASA

The urine ^{14}C activity was much higher after instillation of 15 mM ASA into the stomach and reached a maximal value during the second hour in all experiments (Table II) in the 6 h experiments there was a progressive decrease from the second hour onwards (Fig 2)

The urine flow was approximately constant during all the sampling periods

TABLE I The C activity in the urine and urine flow (ml/h) after i v injection of 1 25 μCi C-inulin C activity in the urine in per cent of the injected activity Mean values ± S E are given n = 3

Hour	1	2	3	4	5	6
^{14}C activity	59 3±0 3	11 9±1 1	5 6±0 7	3 2±0 3	1 7±0 4	1 0±0 3
Urine flow	0 8±0 1	0 8±0 1	0 9±0 2	1 1±0 3	1 0±0 1	1 0±0 2

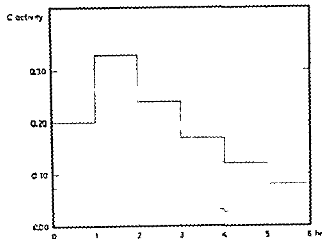


Fig 2 ^{14}C activity in the urine during a 6 h period after the simultaneous instillation of either ^{14}C-inulin and 15 mM ASA in an isotonic (NaCl) solution (continuous line) or ^{14}C inulin in isotonic NaCl (interrupted line) ^{14}C activity expressed as per cent of original activity in the stomach.

The chromatographic elution profiles of the urinary ^{14}C activities derived from inulin—NaCl or inulin—ASA in the stomach were determined on 3 pooled samples one from all the hourly samples of each of the following (i) the ASA free periods of 3 out of 5 type b expts (ii) the ASA free periods of all the type c expts and (iii) the ASA period of 2 of the type b expts The profiles of which (ii) and (iii) are given in Fig 3 all had their peaks in roughly the same positions and shifted towards lower molecular values compared with those of the instilled inulin fraction Further in each pooled sample more than 95 percent of the ^{14}C activity was associated with species of molecular weights greater than about 2000

TABLE II The ^{14}C activity in the urine (in per cent of original activity in the stomach) and the urine flow (ml/h) during two consecutive 3 h instillations of ^{14}C inulin in isotonic NaCl and ^{14}C inulin in 15 mM ASA (NaCl to isotonicity) respectively The inulin was either instilled simultaneously with or 20 min after the NaCl or ASA Mean values ± S E are given

Simultaneous instillation (n = 5)

	Inulin in NaCl			Inulin in 15 mM ASA		
Hour	1	2	3	4	5	6
C in urine	0.08? ± 0.013	0.094 ± 0.070	0.063 ± 0.010	0.248 ± 0.0?0	0.3?0 ± 0.038	0.?66 ± 0.04
Urine flow	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1

Inulin after 20 min (n = ?)

	Inulin in NaCl			Inulin in 15 mM ASA		
Hour	1	2	3	4	5	6
C in urine	0.014 0.004	0.0?1 ± 0.004	0.018 ± 0.00?	0.? ? ± 0.049	0.49 0.14	0.?5 ± 0.09?
Urine flow	0.8 0.7	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0	1.0 ± 0.1	1.0 ± 0

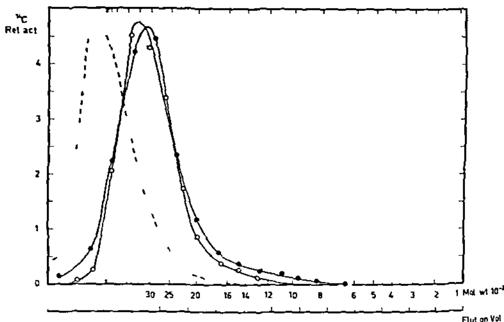


Fig 3 Effluent ^{14}C activity profiles of reduced urine samples after elution through a column of Sephadex G 25 The results were obtained on intragastric instillation of ^{14}C-inulin 90 min after instillation of isotonic NaCl solution (filled circles) and on simultaneous instillation of ^{14}C inulin and 15 mM ASA in an isotonic (NaCl) solution (open circles) The interrupted line is the pH 7 curve of Fig 1 The ^{14}C activities have been arbitrarily adjusted to give the same height as the curve from Fig 1

During the 3 h expts the volumes of the gastric contents increased from 6 to 7 8 ± 0 5 ml and from 6 to 7 6 ± 0 5 ml with and without ASA respectively The respective ranges in terminal pH values were 1 54—5 77 and 1 72—6 54 The inulin losses from the stomach were small and this is reflected in the nearly complete recoveries of ^{14}C activity in the stomachs at the end of the experiments Thus 3 h after the simultaneous instillation of NaCl and inulin 94 ± 5 percent of the ^{14}C activity was still present in the stomach while after ASA and inulin 94 ± 3 percent remained In only one case was the terminal solution blood stained and this was the only experiment in which the hemoglobin concentration was greater than 3 mg/liter This stomach also had macroscopic mucosal erosions

Discussion

ASA increased the ^{14}C activity in the urine The maximum during the second hr and the subsequent decline (Table II Fig 2) are probably correlated with both the transit time for transport of inulin from the stomach to urine and the time of action in and disappearance time of ASA from the stomach A single instillation of ASA into the stomach produced a maximal effect on ion permeability within one hr (Davenport 1964 Frenning 1971) The delay before the ^{14}C activity maximum appeared in the urine depended probably largely on the transit time across the

gastric mucosa since blood—urine transport is rapid (Table I) It is possible however that it is not only the mean time required for migration across the mucosa but for the first molecules to get across which is important since there may be some latency due to the time required for the full effect of ASA on saccharide permeability to develop Both these delay factors probably operate in the frog gastric mucosa *in vitro* (Flemström and Marsden 1973)

ASA escapes rapidly from the gastric lumen (Hogben *et al* 1957 Schanker *et al* 1957 Davenport 1964) very little remained 1 h after instillation of 6 ml 16 5 mM ASA into the cat stomach (Frenning 1971) The declining ^{14}C activity in the urine after the second hr is probably therefore due to a reduction of the ASA induced permeability increase caused by the loss of ASA from the lumen *i e* the ASA effect is reversible

There was some transport of ^{14}C activity into the urine even in the absence of ASA In order to test whether this was due in some way to the initial conditions *i e* it occurred in response to the instillation of fluid into the stomach the type (c) experiment was made. ^{14}C-inulin was not added until 20 min after instillation of NaCl solution This reduced the urinary ^{14}C activity fourfold and we must conclude that the gastric permeability is increased temporarily by the filling procedure In this case the maximal urinary ^{14}C activity is also obtained during the second hr It seems plausible that this permeability increase may be due to stretching of the mucosa and it may have some significance as regards stimulation of secretion where the permeability of the mucosa to different molecules is of importance (Andersson and Elwin 1971 Berkowitz *et al* 1971)

The primary aim of these experiments was to determine if molecules sufficiently large to be antigenic crossed the mammalian gastric mucosa after exposure to ASA Dextran oligosaccharides with more than 10 anhydroglucose residues (mol wt 1800) have been reported to elicit immune reactions in the guinea pig (Richter 1972) The present results indicate that intragastric ASA greatly increases the absorption of molecules of such size or larger Inulin was used here as the test substance instead of dextran as it is rapidly excreted in the urine in quantities sufficient for molecular weight distribution analysis and also essentially inert in the body

In using ^{14}C labelled inulin as an indicator of gastric permeability molecular weight distribution analysis is essential to remove two potentially serious sources of error Firstly inulin hydrolyses if the pH is too low *i e* 1 5 a value approached in the gastric contents in some of our experiments The possibility that the ^{14}C activity in the urine is associated predominantly with low molecular weight fragments must thus be excluded and molecular weight distribution analysis proved here that the intragastric conditions were not sufficiently acid to cause significant hydrolysis Secondly only a relatively small fraction of the inulin instilled into the stomach was absorbed and thus the presence of significant amounts of radiochemical impurities such as low molecular ^{14}C containing fragments in the urine must also be excluded Carbohydrate polymers are very liable to radiolytic self-decomposition (*cf* Bayly and Weigel 1960) It should also be noted that the carboxyl ^{14}C inulin is end group

labelled and thus the ^{14}C activity in the urine is proportional to the number of molecules present and not to the weight of inulin as would be the case with a 'randomly' labelled inulin This is of no importance in the present context where molecular weight distribution analyses were made

The use of a polydisperse polymer with molecular weight distribution analysis has the *advantage that with the proper polymer the penetration* limits can be established in a single experiment (Flemstrom and Marsden 1973) In this respect inulin has the disadvantage that its upper size limit is at a mol wt of about 10 000 and hence well below the ASA induced permeability limits found in the frog gastric mucosa *in vitro* (Flemstrom and Marsden 1973) This limitation, however is unimportant here since as mentioned above the aim of these experiments was to establish that molecular species sufficiently large to be antigenic could be absorbed from the gastric lumen after ASA and not to define a change in the upper permeability limit Since the latter is probably above the size range of inulin another polymer would be required for its determination

Although the gel G 25 cannot resolve the upper molecular weight region of the original inulin fraction a small shift in the molecular weight maximum in the urinary ^{14}C activity is evident and this indicates a moderate degree of molecular sieving (Fig 3) Since this distribution after gastric instillation is similar to that found after intravenous injection it indicates further that the sieving must be due mainly at least to the glomerular membrane and it is very probable that the cat gastric mucosa is permeable to considerably larger molecules than were tested here It was also found that the molecular weight distribution of the urinary inulin after instillation of ASA was about the same as that in the much smaller amounts recovered with intragastric isotonic NaCl alone This is not surprising since the molecular sieving of inulin seems to be mediated predominantly by the glomerular membrane

With one exception bleeding was not detected during the present experiments This is in agreement with earlier reports that ASA causes bleeding only if there is simultaneously a very low intraluminal pH (Davenport 1969) or stimulation of acid secretion (Davenport 1965)

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References

ANDERSSON S and C F ELWIN Relationship between antral acidity and gastrin releasing potency of chemical stimulants *Acta physiol scand* 1971 *83* 437—445
BAYLY R J and H WEIGEL Self decomposition of compounds labelled with radioactive isotopes *Nature* (Lond) 1960 *188* 384—387
BERKOWITZ J M G BUETOW M WALDEN and M PRAISSMAN Molecular factors in antral permeation their proposed role in gastrin release *Amer J Physiol* 19[illegible] [illegible] 259—265
BRAY G A A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter *Analyt Biochem* 1960 *1* 279—285
DAVENPORT H W Gastric mucosal injury by fatty and acetylsalicylic acids *Gastroenterology* 1964 *46* 245—253

DAVENPORT H W Damage to gastric mucosa effects of salicylates and stimulation *Gastroenterology* 1965 *49* 189—196
DAVENPORT H W Fluid produced by the gastric mucosa during damage by acetic and salicylic acids *Gastroenterology* 1966 *50* 487—499
DAVENPORT H W Salicylate damage to the gastric mucosal barrier *New Engl J Med* 1967 *276* 1307—1312
DAVENPORT H W Gastric mucosal haemorrhage in dogs Effects of acid aspirin and alcohol *Gastroenterology* 1969 *56* 439—449
DAVENPORT H W Protein losing gastropathy caused by sulphydryl reagents *Gastroenterology* 1971 *60* 870—879
EGGLETON M G and Y A HABIB The mode of excretion of creatinine and inulin by the kidney of the cat *J Physiol* (Lond) 1951 *112* 191—200
FLEMSTROM G B FRENNING and K J OBRINK The disappearance of acetic acid and acetate from the cat's stomach and its influence on the permeability of hydrochloric acid *Acta physiol scand* 1964 *62* 422—428
FLEMSTROM G and B FRENNING Migration of acetic acid and sodium acetate and their effects on the gastric transmucosal ion exchange *Acta physiol scand* 1968 *74* 521—532
FLEMSTROM G Intracellular accumulation and permeability effects of some weak acids in the isolated frog gastric mucosa *Acta physiol scand* 1971 *82* 1—16
FLEMSTROM G and N V B MARSDEN Dextran permeability electrical properties and H secretion in isolated frog gastric mucosa after acetylsalicylic acid *Gastroenterology* 1973 *64* 278—284
FRENNING B The disappearance of acetylsalicylic acid (ASA) in aqueous solution from the cat stomach and its influence on the transmucosal ion transport in the innervated non secreting cat stomach *Acta physiol scand* 1971 *83* 235—246
GALDINO M I L SCHWARTZ and M F LEVITT Inulin volume of distribution as a measure of extracellular fluid in dog and man *Proc Soc exp Biol* (N Y) 1948 *68* 507—511
HEIDELBERGER M A C AISENBERG and W Z HASSID Glycogen an immunologically specific polysaccharide *J exp Med* 1954 *99* 343—353
HOGBEN C A M L S SCHANKER D J TOCCO and B B BRODIE Absorption of drugs from the stomach II The human *J Pharmacol exp Ther* 1957 *120* 540—545
IVEY K J Gastric mucosal barrier *Gastroenterology* 1971 *61* 247—257
KABAT E A and D BERG Dextran — an antigen in man *J Immunol* 1953 *70* 514—532
MARSDEN N V B Solute behavior in tightly cross-linked dextran gel *Ann N Y Acad Sci* 1965 *125* 428—457
RICHTER W Minimal molecular size of dextran required to elicit heterologous passive cutaneous anaphylaxis in guinea pigs *Int Arch Allergy* 1972 *43* 252—268
SAMTER M and R F BEERS Intolerance to aspirin Clinical studies and consideration of its pathogenesis *Ann intern Med* 1968 *68* 975—983
SCHANKER L S P A SHORE B B BRODIE and C A M HOGBEN Absorption of drugs from the stomach I The rat *J Pharmacol exp Ther* 1957 *120* 528—539
SMITH B M J J SKILLMAN G B EDWARDS and W SILEN Permeability of the human gastric mucosa Alteration by acetylsalicylic acid and ethanol *New Engl J Med* 1971 *285* 716—721

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From the Institute of Physiology, University of Helsinki Finland

A Serum Factor Influencing the Physiological Activity of Thyrotropin-Releasing Hormone (TRH)
II Studies with rat serum in vivo and in vitro

By

Pekka Virkkunen Harry Lybeck, Tapio Ranta and Juhani Leppäluoto

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Abstract

Virkkunen P H Lybeck T Ranta and J Leppäluoto *A serum factor influencing the physiological activity of thyrotropin releasing hormone (TRH) II Studies with rat serum in vivo and in vitro* Acta physiol scand 1974 92 526–529

The mid fractions in gel filtration of rat serum proteins were shown to be the most potent in abolishing the biological activity of TRH during incubation in vitro The kidneys were not able in vitro or in vivo to restore the biological activity of inactivated TRH Biologically active TRH labelled TRH and serum inactivated labelled TRH were excreted in the urine in equal amounts

It is well known that the biological activity of TRH is abolished by incubation with serum at body temperature Two inactivation mechanisms have been suggested

1 enzymic deamination of TRH or

2 complexing of TRH to a serum protein

Virtually all the experimental evidence to date supports the first alternative (Redding and Schally 1968 Nair Redding and Schally 1971 Bassiri and Utiger 1972 Virkkunen 1974 a)

TRH whether radioactive or not is excreted in large amounts in urine (Guillemin 1971, Redding and Schally 1971 Virkkunen Leppaluoto and Lybeck 1972, Leppaluoto Virkkunen and Lybeck 1972) It is not known at present to what extent this excretion interferes with the estimation of the inactivation of TRH in vivo

In the present study using ¹⁴ I labelled TRH unlabelled TRH and bioassay we found that TRH does not form complexes with serum proteins We infer that serum inactivation of TRH and excretion of TRH in urine are independent phenomena in the rat

Material and Methods

Inactivation of TRH with serum. TRH (Ferring Ltd) was added (36 ng in 20 μl of saline) to the following freshly prepared solutions: 1.2 ml rat serum, 1.2 ml saline, 1.2 ml serum and saline dilution and 1.2 ml serum fractions (see below). The test tubes were agitated in a water bath at 37° C for 0, 10, 30 and 90 min. At the end of the incubation period 0.2 ml aliquots were immediately injected into bioassay mice prepared 24 h beforehand. Rat serum was submitted to gel filtration, and fractions were collected and bioassayed as described in a previous paper (Virkkunen 1974 a). ^{125}I labelled TRH was prepared according to Virkkunen *et al.* (1973).

Administration of TRH or ^{125}I TRH after incubation with rat serum. TRH or ^{125}I TRH was incubated with serum or saline and injected i.v. into 21 rats anesthetized with pentobarbital (30 mg/kg). The TRH content of pooled urine collected 30 min later was determined by a bioassay described in an earlier paper (Virkkunen 1973 b) or by measurement of radioactivity. Samples of ^{125}I TRH directly after incubation and in urine were also submitted to paper electrophoresis (acetic acid buffer, pH 2.5, 200 V, 2 h) or thin layer chromatography (chloroform: methanol: conc. ammonia 60: 45: 20, chloroform: methanol: acetic acid 60: 40: 20). The radioactivity of the paper strips and plates was measured with an automatic scanner (Berthold).

Results

Incubation of TRH with rat serum. TRH was incubated with rat serum for 0, 10, 30 and 90 min at 37° C. The bioassayable TRH content fell significantly in 10 min (Table I).

A 10 ml pool of rat serum was filtered through a Sephadex G 200 column, and four fractions with relative elution volumes of 1—1.3, 1.3—1.6, 1.6—1.9 and 1.9—2.2 were collected. They were incubated with TRH for 90 min at 37° C. The first and last fractions did not abolish the biological activity of TRH, whereas the mid fractions did (Table II).

In vivo administration of TRH or ^{125}I labelled TRH incubated with rat serum or saline. TRH was incubated with rat serum or saline for 90 min. Thereafter aliquots of both incubation media were injected i.v. into rats anesthetized with pentobarbital. 30 min later urine was collected and assayed for TRH content. Rats given TRH incubated with saline had 16 ± 5 % of the dose in their urine, whereas no biological activity ($p < 0.01$) was found in those given TRH incubated with rat serum (Table III). Serum inactivated TRH was also reincubated with whole kidney homogenate but no biological activity was found in the supernatant after reincubation. The bioassay response before reincubation was 1.91 ± 0.02 and after it 1.91 ± 0.02 (not seen in the table).

^{125}I TRH was incubated with rat serum or saline and injected into rats as before. 30 min later urine was collected and its radioactivity measured. The urine contained 18 ± 4 % of the dose given after incubation with saline and 21 ± 3 % of that given after incubation with serum (Table III).

In low voltage paper electrophoresis (acetic acid, pH 2.5) and in thin layer cromatography on Silicagel with 2 buffers ^{125}I labelled TRH formed one peak which remained unchanged after incubation with rat serum or after administration in vivo.

TABLE I Bioassay responses of 6 ng TRH after incubation with rat serum or saline for various periods

Treatment: Incubation at 37 C	Time	Number of mice	Bioassay response ($\log y \pm \log x+2$) mean ±S E
saline	0	6	2 51±0 10
rat serum	0	6	2 40±0 0ɔ NS
	10 min	7	1 97±0 0?**
	30 min	7	1 96±0 03 *
	90 min	7	1 9ɔ±0 01**

* = $p < 0\ 0ɔ$ ** = $p < 0\ 01$ and NS = $p > 0\ 0ɔ$ from the saline control

TABLE II Bioassay responses of 6 ng TRH after incubation with various fractions of rat serum

Treatment: Incubation at 37 C	Time	Number of mice	Bioassay response ($\log y \pm \log x+2$) mean±S E
saline	0	5	2 09±0 0ɔ
fraction 1 0–1 3	90 min	5	2 07±0 0?NS
1 3–1 6		5	1 91±0 04
1 6–1 9		5	1 91±0 03
1 9–2 2		5	2 11±0 03NS

For explanations see Tabl 1

TABLE III Recovery of TRH and ^{125}I TRH in rat urine after preincubation with saline or serum

Pre incubation media	Number of rats	TRH detected in urine at 30 m n	
		Biological activ	Rad oactivity
Experiment with TRH			
saline	6	16±4	—
serum	6	below 2 5 *	—
Experiment with ^{125}I TRH			
saline	4	—	18±4
serum	5	—	21±3 NS

= percentages of the dose of TRH for other explanations see Table I

Discussion

In agreement with the original findings of Redding and Schally (1968) we demonstrated here that rat serum abolished the biological activity of TRH during incubation at 37° C. We were also able to show that the mid fractions of the serum proteins were the most potent in this respect as in human serum (Virkkunen 1974 a).

The present experimental evidence suggests that TRH is inactivated through deamination of proline amide both in vitro (Nair, Redding and Schally 1971) and in vivo (Redding and Schally 1971). The rapid accumulation of TRH in urine in vivo promoted us to test the ability of the kidney to reactive serum inactivated TRH. But neither in vitro nor in vivo were the kidneys able to restore the biological activity of inactivated TRH.

Biologically active TRH labelled and serum inactivated labelled TRH were excreted in the urine in equal amounts (Table III) and even had similar mobilities in electrophoresis and thin layer chromatography. However biological activity was totally abolished by incubation with serum in vitro. These findings strongly support the view that TRH is inactivated by serum through deamination because deamination produces only slight physico-chemical changes but deaminated TRH is not biologically active (Burgus *et al.* 1969).

In our thin layer chromatography and electrophoresis systems it was not possible to distinguish labelled TRH from TRH incubated with serum. This seems to be an unsolved problem also elsewhere.

The kidneys do not appear to differentiate between TRH and inactivated TRH and do not reactivate the latter although TRH is found in large amounts in the urine.

References

Bassiri R. and R. Utiger, Serum inactivation of the immunological and biological activity of thyrotropin releasing hormone (TRH). *Endocrinology* 1972. *91*. 657—664.

Burgus R., D. Dunn, D. Desiderio and R. Guillemin, Structure moléculaire du facteur hypothalamique hypophysiotrope TRF d'origine ovine. *C. R. Acad. Sci.* (Paris) 1969. *269*. 1870—1873.

Guillemin R., Thyrotropin releasing factor. *Proc. Int. Union Physiol. Sci.* 1971. *8*. 257—258.

Leppaluoto J., P. Virkkunen and H. Lybeck, Elimination of TRH in man. *J. clin. Endocr.* 1972. *35*. 477—478.

Nair, R. M. G., T. W. Redding and A. V. Schally, Site of inactivation of thyrotropin releasing hormone by human plasma. *Biochemistry* 1971. *10*. 3621—3624.

Redding T. W. and A. V. Schally, Studies on the inactivation of thyrotropin releasing factor (TRF). *Fed. Proc.* 1968. 7 8 3.

Redding T. W. and A. V. Schally, The distribution of radioactivity following the administration of labeled TRH in rats and mice. *Endocrinology* 1971. *89*. 1075—1079.

Virkkunen P., J. Leppaluoto and H. Lybeck, The distribution and elimination of [125]I labelled thyrotropin releasing hormone in the mouse. *Horm. Met. Res.* 1972. *4*. 506—507.

Virkkunen P., J. Leppaluoto, T. Ranta and H. Lybeck, Preparation of [125]I labelled thyrotropin releasing hormone. *Horm. Met. Res.* 1973. 5. 58.

Virkkunen P., A serum factor influencing the physiological activity of thyrotropin releasing hormone (TRH). I. Studies with human serum. *Acta physiol. scand.* 1974 a. *9*. 416—420.

Virkkunen P., Some factors influencing TRH bioassay in mice. *Acta endocr.* (Kbh.) 1974 b. 75. 457—460.

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From the Department of Toxicology University of Uppsala, Sweden

Autoradiographic - Histochemical Demonstration of Tyrosinase Activity in Melanin-forming Tissues of Mammals

By

NILS GUNNAR LINDQUIST

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Abstract

LINDQUIST N G *Autoradiographic histochemical demonstration of tyrosinase activity in melanin forming tissues of mammals* Acta physiol scand. 1974 92 530—536

The tyrosinase activity in melanin forming tissues was investigated in a series of rats (6 days to 1 year old one cat 30 days old and one 4 month old human fetus. The technique used was an autoradiographic-histochemical method utilizing the ^{14}C-labelled melanin precursor tyrosine as a substrate A high accumulation was found in the iris and ciliary body in the young pigmented animals and also in the human fetus In addition a rather high activity was observed in the choroid of these specimens When sodium diethyldithiocarbamate (10 M) a tyrosinase inhibitor was added to the incubation solution no specific uptake was seen. The level in the corresponding tissues of the albino rats was low In the 1 year old pigmented rat a significant activity was still seen in the uveal tract and was very pronounced in the iris—indicating that the melanin formation in the eye may continue even into the adult stage High tyrosinase activity was also found in other melanin forming tissues such as those in the inner ear and the hair follicles of the human fetus and in the hair follicles skin, and meninges of the young pigmented rats

Key words
Tyrosinase melanin formation eye inner ear skin hair follicles autoradiograph histochemistry

Mammalian tyrosinase has been found to catalyze the formation of melanin from tyrosine (Fitzpatrick *et al* 1950 Fitzpatrick and Kukita 1959 Seiji and Fitzpatrick 1961) When sections (or tissue pieces) are incubated in a buffer containing tyrosine or 3 4-dihydroxyphenylalanine (DOPA) newly formed melanin is deposited in the cytoplasm of cells with tyrosinase activity (Bloch 1916 Becker *et al* 1935 Fitzpatrick *et al* 1950) The reaction product melanin is strongly bound to a cytoplasmatic organelle the melanosome which contains the tyrosinase (Seiji and Fitzpatrick 1961 Tyrosinase activity has been demonstrated in melanocytes in the skin the hair follicles and in the eyes of embryos and fetuses (Bloch 1916 Miescher 1922 Becker *et al* 1935 Fitzpatrick *et al* 1950 Miyamoto and

Fitzpatrick 1957 Brunet and Small 1959 Fitzpatrick and Kukita 1959 Toda and Fitzpatrick 1971, Lerner and Hendee Jr 1973) Miescher (1922) Miyamoto and Fitzpatrick (1957) and Fitzpatrick and Kukita (1959) did not find any enzymatic activity in the adult mammalian eye

In previous investigations in pigmented mice with autoradiographic histochemistry (using ^{14}C labelled tyrosine as a substrate) the highest tyrosinase activity in the body was found in the eyes of fetuses (Lindquist 1973) Strong activity was also observed in the iris of the adult pigmented mouse

The present study on tyrosinase activity in the mammalian body was extended to some other species A series of rats (6 days to 1 year old) one cat 30 days old and one 4 month old human fetus were used Whole body sections from the young rats and the human fetus and sections through the head of the 1 year old rat and the young cat were incubated in a buffer containing ^{14}C tyrosine and then autoradiographed

Materials and methods

L-tyrosine ^{14}C uniformly labelled with a specific activity of 522 mCi/mmol was obtained from The Radiochemical Centre Amersham England The tyrosinase inhibitor sodium diethyldithiocarbamate was purchased from Fluka AG Buchs Switzerland

5 pigmented rats (6 12 18 28 days and 1 year old) of the Lister strain and four albino rats (6 12 18 and 28 days old) of the Sprague Dawley strain were used The animals were kept on a complete pellet diet (AB Ewos, Sodertalje Sweden) at a room temperature of +25 C with free access to water

One 30 days old pigmented cat and one 4 month old Caucasian human fetus weight 100 g crown heal length 18 cm) were also used

The animals were killed with chloroform anesthesia The 1 year old rat and the cat were decapitated The young rats the human fetus and the heads of the adult rat and cat were mounted in a gel of carboxymethyl cellulose and immersed in n hexane cooled to − 8 C with solid carbon dioxide Sagittal sections 20 μm thick were cut (according to Ullberg's technique 1954 1958) through the whole body of the young rats and the human fetus and through the heads of the adult rat and cat Immediately after the sections had been cut they were fixed for about 15 s in a supercooled 3 5 % formaldehyde solution adjusted to a pH of 7 4 with a cacodylate buffer at −15 C before they were allowed to attain room temperature in an airtight box

The autoradiographic histochemical method of Fitzpatrick and Kukita (1956) as modified for tape mounted sections The incubation solution consisted of 1 μCi L-tyrosine ^{14}C corresponding to 0 4 μg tyrosine that we added to 10 ml of either a 0 1 M phosphate buffer solution pH 6 8 or a 0 1 M cacodylate buffer pH 7 4 The incubation time was 2 h at +37 C.

As controls representative sections from each specimen were treated identically as above except that the tyrosinase inhibitor Na-diethyldithiocarbamate (10 M) was added to the incubation solution

After incubation the sections were rinsed in running water for 1 h and air-dried Autoradiograms were made by apposition of the sections against roentgen films (Crystallex Kodak) The times of exposure varied from 3 weeks up to 7 months After exposure the sections were separated from the films The films were processed with Gevaert G 150 developer and Gevaert G 305 fixative A number of sections were stained in hematoxylin and eosin and mounted in Euparal® (Flatters & Garnett Ltd Manchester England

Results

Following incubation of whole body sections of young rats and a 4 month old human fetus and sections through the heads of a 30 days old cat and a 1 year old rat—in a buffer containing ^{14}C tyrosine—a high activity was observed in

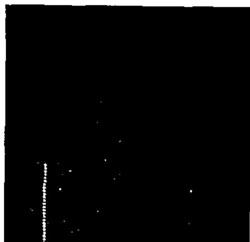
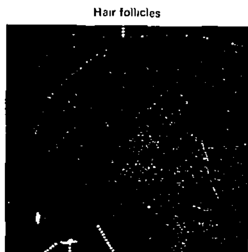


Fig 1 Detail of an autoradiogram of a whole body section of a 28 days old albino rat The section was incubated in a buffer containing ^{14}C tyrosine There is no evidence of specific uptake in the uveal tract.

melanin forming tissues The level in the corresponding tissues of albino rats was low (Fig 1) A very high uptake was seen in the eye The activity in the eye decreased with age When sodium diethyldithiocarbamate (10 M) a tyrosinase inhibitor was added to the incubation solution no specific uptake was seen The uptakes in the various melanin forming tissues will be described in detail below

Eye In the young rats (6 12 18 and 28 days old) a high accumulation was observed in the iris and the ciliary body (Fig 2 3) Rather high activity was also



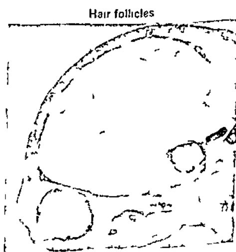


Fig 2 Detail of an autoradiogram (left) of a whole body section (right) of a 6 days old pigmented rat The section was incubated in a buffer containing ^{14}C tyrosine There is high activity in the iris and in the ciliary body Rather high uptakes can b[e seen in] the choroid and in the hair follicles Hematoxylin eosin

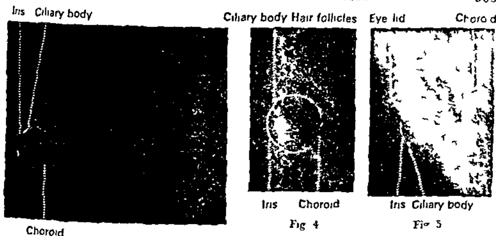


Fig 3 Fig 4 Fig 5

Fig 3 Detail of an autoradiogram of a whole body section of a 28 days old pigmented rat The section was incubated in a buffer containing ^{14}C tyrosine There is high accumulation in the iris and in the ciliary body Rather strong activity is also seen in the choroid

Fig 4 Detail of an autoradiogram of a section of a 30 days old pigmented cat The section was incubated in a *buffer containing ^{14}C tyrosine The highest uptake within the uveal tract* is seen in the iris

Fig 5 Detail of an autoradiogram of a section of a 1 year old pigmented rat The section was incubated in a buffer containing ^{14}C tyrosine There is a rather high activity in the iris A specific uptake is also seen in the ciliary body in the choroid and in the eye lid

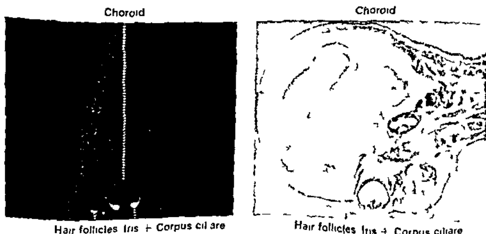


Fig 6 Detail of an autoradiogram (left) of a whole body section (right) of a 4 month old Caucasian human fetus The section was incubated in a buffer containing ^{14}C tyrosine There is high accumulation in the iris and in the ciliary body High activity is also seen in the choroid and in the hair follicles Hematoxylin-eosin

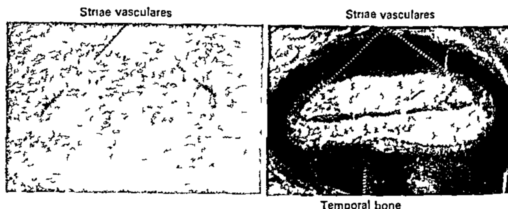


Fig 7 Detail of an autoradiogram (left) of a whole body section (right) of a 4 month old Caucasian fetus The section was incubated in a buffer containing ^{14}C tyrosine There is high activity (black grains) in the stria vascularis of the cochlea Hematoxylin eosin (×10)

seen in the choroid In the 30 days old cat the highest uptake within the uveal tract was observed in the anterior part of the eye (Fig 4) In the 1 year old rat there was a weaker but still significant activity in the uveal tract very pronounced in the iris (Fig 5)

In the 4 month old human fetus there was a high accumulation in the iris and the ciliary body (Fig 6) A high uptake was observed also in the choroid

Inner ear A high activity was seen in the striae vasculares (Fig 7) and in the walls of the ampullae of the 4 month old human fetus Very low activity was found in the inner ear of the young rats and the young cat The autoradiograms from the 1 year old rat were difficult to interprete concerning accumulation in the inner ear because of the difficulty in obtaining good sections through the hard temporal bone

Skin and hair follicles There was a high activity in the eye lids of the rats and the 30 days old cat There was no specific uptake in the skin of the human fetus In the hair follicles there was a high accumulation in the rats (Fig 2) the cat (Fig 4) and the human fetus (Fig 6)

Meninges A rather high activity was found in the meninges of the young rats up to 28 days of age No accumulation was found in the meninges of the human fetus

Discussion

The tyrosinase activity in some mammalian tissues such as in the skin and in the hair follicles has been throughly investigated Fitzpatrick *et al* (1950) showed that ultraviolet irradiation stimulates tyrosinase activity in the melanocytes of exposed human skin Fitzpatrick and Kukita (1959) demonstrated that the enzymatic ac

tivity in the melanocytes in the hair bulb is high during the growth phase of the hair cycle. Also in the eye the tyrosinase activity has been found to be high during growth. Strong enzymatic activity has been found in the eyes of embryos and fetuses (Miescher 1922, Miyamoto and Fitzpatrick 1957, Fitzpatrick and Kukita 1959). These authors found no enzymatic activity in the adult mammalian eye. It has been suggested that the pigment cells in the eye have only a limited period of melanin formation and once they have produced all their melanosomes—before birth—they retain them throughout life (Masson 1948).

In a previous investigation in our laboratory the highest tyrosinase activity in the pigmented mouse was found in the fetal eye (Lindquist 1973). The enzymatic activity in the eye decreased with age but strong activity was still present in the iris in the 3 month old mouse. In the present study the same pattern was found in other species. Very high tyrosinase activity was present in the eye of a 4 month old human fetus. In a 30 days old cat the highest enzymatic activity in the eye was observed in the iris and in the ciliary body. Also in young rats (6—28 days old) high tyrosinase activity was found in these structures and in a 1 year old rat strong activity was noted in the iris. These results indicate that the melanin formation in the eye may continue, although to a slight degree, even into the adult stage. However, the uveal tract of pigmented animals has been found to retain drugs with a melanin affinity for a very long time (Potts 1962, Lindquist and Ullberg 1972, Lindquist 1973). This observation indicates that the melanin formation in the eye of adult mammals is low.

In this investigation high tyrosinase activity was also observed in other melanin forming tissues such as in the inner ear and in the hair follicles of the human fetus and in the hair follicles, skin and meninges of the young pigmented rats.

In the literature little attention has been paid to the melanin-containing cells in the inner ear. These cells may play a role in the development of inner ear lesions caused by ototoxic drugs. Chloroquine, an ototoxic drug with melanin affinity, was found to accumulate in the melanin-containing cells in the inner ear of young pigmented rats (Dencker and Lindquist 1974). In the inner ear, melanin producing cells are found in 2 epithelial structures: the stria vascularis in the cochlea and the planum semilunatum in the ampullae (Corti 1851, Alexander 1901, Beck 1961, Sieber and Schmidt 1962, Savin 1965). Melanocytes are also present in the connective tissue in several zones of the labyrinth. A high tyrosinase activity was observed in the striae vasculares and in the walls of the ampullae of the 4 month old human fetus. Very low activity was found in the inner ear of the young rats and the 30 days old cat—which indicates that in these species the melanin formation is already very low in the inner ear short after birth.

This investigation was supported by a grant from The Swedish Board for Technical Development.



Fig 7 Detail of an autoradiogram (left) of a whole body section (right) of a 4 month old Caucasian fetus The section was incubated in a buffer containing ^{14}C tyrosine There is high activity (black grains) in the stria vascularis of the cochlea Hematoxylin eosin (×10)

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From the Laboratory for the Theory of Gymnastics August Krogh Institute University of Copenhagen Denmark

Apparent Efficiency and Storage of Elastic Energy in Human Muscles during Exercise

By

ERLING ASMUSSEN and FLEMMING BONDE PETERSEN

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Abstract

ASMUSSEN E and F BONDE PETERSEN *Apparent efficiency and storage of elastic energy in human muscles during exercise* Acta physiol scand 1974 92 537—545

3 subjects ran on the treadmill (10 km/h) against varying horizontal impeding forces One subject was further studied during the same kind of walking and bicycling on the treadmill and during work consisting in lowering and lifting the body by flexing and extending the legs from a standing or sitting position at varying frequencies with or without rebound in the deepest position Workpower (W kcal/min) and the corresponding steady state metabolic rate (E kcal/min Douglas bag method) were measured Apparent efficiency (N) was calculated as $\Delta W/\Delta E \times 100$ % During load running N was 53.8 37.6 and 41.2 % respectively in the 3 subjects In the subject more extensively studied N was running 53.8 walking 32.3 bicycling 25.1 knee flexions (deep or half) with rebound 39.4 or 41.0 without rebound 26.1 or 21.9 % These variations in N % were explained in accordance with the possibilities for re using the energy absorbed and stored in the muscles as elastic energy during a phase of negative exercise in a subsequent phase of positive exercise The condition of this is that the positive phase follows immediately after the negative A calculation showed that during running 35—53 % of the energy absorbed during the negative phase was re used Corresponding figures for walking and rebounding knee extensions were 23 and 34 % respectively while in bicycling and knee extensions without rebound all of the negative work degenerated into heat

The possibility that implanted mechanical energy may be temporarily stored in the series elastic components of active muscles for re use in a following contraction was investigated in a previous study of the vertical jump (Asmussen and Bonde Petersen 1974) As discussed in that paper one necessary condition of such a possibility is that the muscles are being forcibly stretched while actively resisting movement *i e* are performing eccentric contractions and doing negative work Another is that the phase of negative work is followed immediately by a phase of positive work—*i e* a phase in which the muscles are shortening Such conditions are realized in running

TABLE I Personal data of subjects

Subject	Age	Height cm	Weight kg
OM	25	174	73
MS	30	175	77
RH	33	170	79

as pointed out *e g* by Fenn (1930) and more recently by Cavagna *et al* (1964), but also in other activities as bouncing up and down by flexing and extending hips and knees (Thys *et al* 1972)

If re use of negative work takes place in exercise comprising alternating positive and negative work one would expect that the amount of metabolic energy set free in the positive work phase were less than when the same amount of positive work is performed without re use of negative work Evidence for this has been given in experiments by Thys *et al* (1972) and by Lloyd and Zacks (1972) who demonstrated that the efficiency (apparent efficiency, defined as the reciprocal of b in the equation metabolic rate = constant + b × work power, *i e* as Δ work power/Δ metabolic rate) was considerably higher in neg pos work than in simple positive work

It was the purpose of the present investigation to repeat and expand experiments the same general nature as those of Lloyd and Zacks and of Thys *et al* in an to add further evidence to the hypothesis The experiments were performed s running walking or bicycling exercise on the horizontal treadmill with loads of varying weights pulling backwards on the subjects and as standing respectively sitting kneebendings and extensions with varying frequencies and loads on the subjects

Methods

As subjects served 3 men whose relevant data are presented in Table I OM was of average physical fitness a skin diver but with no training as a runner MS was of good fitness an *experienced middle and long distance runner without being in top class* RH of good fitness as a cyclist had no recent experience in endurance running

I Running walking and bicycling on the treadmill These experiments were planned and executed in much the same way as those of Lloyd and Zacks The subject ran walked or cycled *on the treadmill with a string attached to* a broad belt of canvas or to the saddle of the bicycle The string passed horizontally over a pulley at the wall behind *the* subject and carried a hook that could be loaded with known weights In this way a well defined constant horizontal resistance had to be overcome by the subject who therefore *performed* work in excess of unloaded running or cycling at the same speed The increase in workpower was (load in kg) × (velocity in m/min) This extra power (W) was *expressed* as kcal/min (1 kcal = 427 kpm) The subject was furnished with mouthpiece and lowresistance respiratory valves so that expired air could be collected in Douglas bags placed *on a metal* net directly over the subject Two 150 l bags were nearly filled in direct succession after the subject had reached a steady state This was assumed to have taken place after *6—8 min* of exercise The contents of the bags were measured by means of a large Tissot *gas meter* and analyzed for CO and O on an infra red CO analyzer (Capnograph Beckman LB 1) and a paramagnetic O analyzer (Servomex type DA 181) These electronic analyzers were *frequently* checked by

TABLE II Data for the regression equations of the results on load running plotted in Fig 1 The constants in the equation $y = a+bx$ the correlation factor r and the apparent efficiency N % calculated as 100/b

Subject	n	a (kcal/min)	b	r	N % apparent efficiency
OM	21	14 81	1 86	0 94	53 8
MS	9	12 22	2 66	0 99	37 6
RH	8	15 50	2 43	0 98	41 2

The results were plotted in a diagram with workpower as abscissa and metabolic rate as ordinate When it had been observed by eye that the data were distributed evenly around a straight line the data were fed into a programmed computer and the regression equation in the form $y = a + bx$ with correlation factor r computed In this equation the inverse value of b is an expression of the apparent efficiency *i e* of $\Delta W/\Delta E$ By multiplication with 100 it was expressed as per cent (N %)

Results

The results from horizontal loaded running on the 3 subjects are presented in Fig 1 The data from the regression equation are presented in Table II as constants in the equation $y = a + bx$ correlation factor r and apparent efficiency the reciprocal of b

It appears from Table II that the 3 subjects differ with respect to horizontal running economy (the constant a) and also to apparent efficiency The differences in a are only partly explained by the different body weights of the subject (Table I) Thus eliminating body weight and expressing running economy as $kcal \times kg^{-1} \times km^{-1}$ it comes out that the difference still exists as OM uses 1 22 MS 0 95 and RH 1 18 $kcal \times kg^{-1} \times km^{-1}$

Apparent efficiency in loaded running—*i e* the efficiency of the extra work performed by the runner on the attached weight—is high in all 3 subjects highest in OM who according to his energy output per kg and km is the least efficient runner The value from subject MS—who was the best trained and most efficient runner—is lowest 37 6 % a value that comes close to the average values found by Lloyd and Zacks (1972) on 3 experienced and welltrained athletes

Subject OM—who had the highest apparent efficiency (53 8 %) in loaded running—was also studied during loaded walking and loaded bicycling on the treadmill The data are shown in Fig 2 (with the regression line from the running experiments of Fig 1 for comparison) and the calculated constants of the regression equations in Table III

It appears from Table III that the a values—*i e* the energy liberation during unloaded bicycling or walking—are quite normal as compared to data from the literature The apparent efficiency for bicycling is also within the limits usually found in bicycling but the efficiency in performing the extra work while walking is remarkably high although less than during running (Table II)

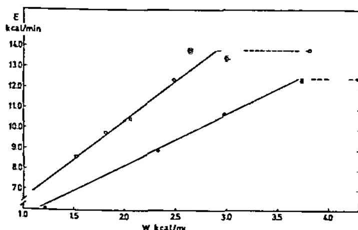


Fig 3 Metabolic rate (E) against work power (W) in rhythmic deep leg flexions and extensions at varying frequencies *with* (closed circles) and *without* (open circles) rebound A larger circle around a point signifies experiments in which the subject carried an extra load weighing 10 kg on the shoulders Dotted lines indicate that a maximum value for oxygen uptake has been reached

TABLE III Data for the regression equations of the results plotted in Fig 2 for subject OM studied during load walking and bicycling The constants in the equation $y = a+bx$ the correlation factor r and the apparent efficiency % calculated as 100 b

Subject OM	n	a (kcal min)	b	r	% apparent fficiency
walking	9	5 28	3 10	0 99	32 3
bicycling	10	? 85	3 99	0 99	25 1

TABLE IV Data for the regression equations of the results plotted in Fig 3 for subject OM studied during knee bending with or without rebound The constants in the equation $y = a+bx$ the correlation factor and the apparent efficiency % calculated as 100 b

			a (kcal min	b		% pparent efficiency
deep knee bending	ebound	10	3 29	? 54	0 9	39 4
	no rebound	11	2 5	3 84	0 9	6 1
half knee bending	rebound	10	3 11	? 44	0 9	41 0
	no rebound	1?	0 85	4	0 9	19

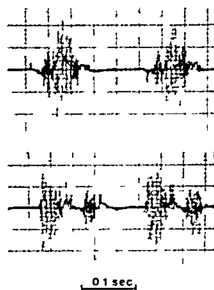


Fig 4 Electromyographic records by surface electrodes placed over m vastus lateralis during down up movements with rebound (upper tracing) and during up down movements (no rebound lower tracing) Note the short period of inactivity in the latter case

The results from the *rebounding experiments* (on subject OM only) are presented as individual data in Fig 3 (deep knee bendings) and as regression constants etc from the straight part of the graphs in Table IV The apparent efficiency was 21 9—26 1 % without rebounce—*i e* within the normal range of efficiencies found *e g* in stair climbing uphill walking and bicycling—whereas the apparent efficiency in the work with rebound was 39 4—41 0 %—considerably higher than the usually accepted values in muscular work At work loads higher than about 3 5 kcal/min the metabolic rate did not increase further This is probably because the subject had reached his maximal oxygen uptake for this kind of exercise

The activity of one of the principal active leg muscles during this kind of exercise is illustrated by the emg s from the vastus lateralis presented as Fig 4 It demonstrates the uninterrupted activity during the down up movements (rebound) and the short period of inactivity at the change of direction in the up down movements

Discussion

Our results from *loaded running* on apparent efficiency are similar to those of Lloyd and Zacks (1972) in that they show very high efficiencies higher than could be expected from biochemical and thermodynamical data (see discussions by Lloyd and Zacks (1972) and Whipp and Wasserman (1969)) Also our data on loaded horizontal walking are surprisingly high whereas loaded horizontal bicycling gave normal values One important difference between the mechanical activity of the leg muscles in running and bicycling is that in running the extensors of the legs perform alternating negative and positive work during the contact period with the ground being stretched during contraction as the body s center of gravity moves

forward downward and shortening and performing work as the center of gravity moves up again This was pointed out e g by Fenn (1930) and by Cavagna et al (1964) These 2 phases—negative to positive—follow immediately upon one another without an intermediate period of muscle relaxation In contrast during bicycling the extensors of the legs are performing positive work during the down stroke but are relaxed during the up-stroke (cf Carlsöo and Molbech 1966) It is therefore quite possible that work performed on the muscles in the negative phase of running, may be stored as elastic energy for re use in the following positive phase This possibility was discussed also by Fenn (1930) who however believed that the saving of energy would be very limited because the muscles to act as springs should maintain tension i e remain in the active state where the elastic stiffness of the fibers is great—and this would cost metabolic energy In running with a stride frequency of 180 strides per minute as in the present experiments each stride lasts 333 ms at 10 km/h About 80 % of this time is spent in contact with the ground (Hogberg 1952) i e about 260 ms Of these 260 ms about half are spent doing negative work i e 130 ms Buchthal and Schmalbruch (1970) found that the time to peak in single twitches in human calf muscles was on an average 74 ms The active state probably lasts close to twice this time It follows that the period of negative work during which elastic energy can be stored is short enough for the active motor units to carry this elastic energy over into the subsequent period of positive work without extra liberation of metabolic energy

The energy implanted in the muscles during the negative phase of the running movements is partly potential gravitational energy and partly kinetic energy In loaded running it is probably only that part of the implanted energy that involves the center of gravity that can be assumed to assist in holding the extra load The work done on the limbs was in the present experiments the same as in unloaded running as the stride frequency was unaltered The work done on the center of gravity is the sum of the vertical lift and the work done in horizontal accelerations of the center of gravity Both Fenn (1930) and Cavagna et al (1964) state that these two are in phase during running (as opposed to walking) and therefore can be summated Consequently the same magnitude of negative work power will be available for re use in the positive work phase of running In loaded running the same conditions probably obtain i e a certain amount of negative work must be available for re use

An estimate of how large a proportion of the extra negative work that can be re used can be made in the following way (cf Fig 1 subject OM) For an increase in aerobic metabolic rate from 14 75 to 18 50 kcal/min —3 75 kcal/min —extra mechanical power corresponding to 2 0 kcal/min is developed Assuming an efficiency for positive work (as on the bicycle Table III) of 25 % the increase in metabolism of 3 75 kcal/min would have led to the performance of only 3 75 × 0 25 = 0 94 kcal/min The difference 2 0—0 94 = 1 06 kcal/min is then so to speak gratis It amounts to 53 % of the work on the load For the 2 other subjects it was 35 % (MS) and 37 % (RH) respectively

These figures are not very different from the estimated value of about 50% elastic energy re used in horizontal unloaded running as calculated by Cavagna *et al* (1964)

In *walking* the work done on the center of gravity also can be divided into work against gravity (vertical lifts) and work performed in changing the kinetic energy of the center of gravity in a horizontal forward backward direction Cavagna and Margaria (1966) who registered and measured this work found that potential work and kinetic energy are opposed in direction the one being positive as the other becomes negative The changes in total energy of the body are therefore small and the negative work that may be available for storing elastic energy is correspondingly small This may explain the lower apparent efficiency of loaded walking as compared to loaded running in the same subject (Fig 2 and Table III) Only about 23% of the work on the load was gratis in loaded walking (as estimated from Fig 2)

The bicycle experiments with extra loads attached, need no long explanations The apparent efficiency of 25% is equal to that found under similar conditions (Bannister and Jackson (1967) Asmussen (1953) Zacks (1973)) and easily explainable in biochemical terms (*cf* Lloyd and Zacks 1972)

The rebounding experiments support the experiments on loaded running in that they show that when rebound is possible the efficiency is considerably higher (N% = 39—41%) than when no rebound takes place (N% = 22—26%) A re use of mechanical potential energy temporarily stored as elastic energy in the active muscles apparently takes place The degree to which this is performed can be estimated from Fig 3 or Table IV as follows (Example from deep knee bending)

With rebound ($y = 3\ 29+2\ 54x$) a positive workpower of say 3 kcal/min will demand extra metabolic energy liberation at the rate of $2\ 54\times3 = 7\ 62$ kcal/min An extra energy liberation of this size would correspond to a mechanical workpower without rebound ($y = 2\ 75+3\ 84x$) of only $7\ 62/3\ 84 = 1\ 98$ kcal/min (plus the same numerical amount of negative work which however will degenerate into heat in the interval between the negative and the positive phase) The performing of 3 kcal/min of positive work with a rebound after negative work of the same numerical value thus has given the difference (3 00—1 98) kcal/min ≈ 1 02 kcal/min without any extra metabolic cost In other words 3 00 kcal/min negative work will turn up after elastic recoil as 1 02 kcal/min positive (and same amount of negative) work 1 02 out of 3 00 kcal represent 34% of the negative work performed In the other series (half knee bending) about 51% of the negative work performed with rebound was saved for the subsequent positive work

There are apparently differences in the subjects ability to re use negative work in running OM seems to be best in this respect in spite of the fact that he was the clumsiest runner It may even be this lack of skill in running that causes this difference maybe because his work against gravity is unnecessarily large On the other hand it may also be due to inherent differences in the structure of the muscles

The present experiments— as those of Cavagna *et al* Thys *et al* Lloyd and Zacks —strongly emphasize the role of muscle elasticity in the economy of muscular

exercise as running jumping etc A recent report by Dawson and Taylor (1973) points to the same energy saving function of the elastic components in the legs of jumping kangaroos Where this elastic component is located—except that it must be in active muscles at least in man—cannot be decided from the present experiments Several reports (Joyce et al (1969), Huxley and Simmons (1971) Wise et al (1973) a o) point to the cross bridges between filaments in the muscle fibers as the most likely location

References

Asmussen E Positive and negative muscular work *Acta physiol scand* 1953 28 364—382

Asmussen E and F Bonde Petersen Storage of elastic energy in skeletal muscles in man. *Acta phys ol scand* 19 4 91 385—392

Bannister E W and R C Jackson The effect of speed and load on oxygen intake for equivalent power outputs during bicycle ergometry *Int Z angew Physiol* 1967 24 284—290

Buchthal F and H Schmalbruch Contraction times and fibre types in intact human muscle *Acta physiol scand* 1970 79 435—452

Carlsoo S and S Molbech The functions of certain two-joint mu cles in a closed muscular chain *Acta morphol neerl scand* 1966 6 377—386

Cavagna G A and R. Margaria Mechanics of walking *J appl Physiol* 1966 21 271—278

Cavagna G A F P Saibene and R. Margaria Mechanical work in running *J appl Physiol* 1964 19 249—256

Dawson T J and C R Taylor Energetic cost of locomotion in kangaroos *Nature (Lond)* 1973 246 313—314

Fenn W O Frictional and kinetic factors in the work of sprint running *Amer J Physiol* 1930 92 583—611

Hoogerwerf P Length of stride stride frequency "flight period and maximum distance between the feet during running with diff rent speeds *Arbeitsphysiologie* 1937 14 431—436

Huxley A F and R M Simmons Mechan cal properties of the crossbridges of frog striated muscle *J Physiol (Lond)* 1971 218 59P—60P

Joyce G C and P M H Rack Isotonic lengthening and shortening movements of cat soleus muscle *J Physiol (Lond)* 1969 204 475—491

Joyce G C P M H Rack and D R Westbury The mechanical properties of cat soleus muscle during controlled lengthening and shortening movements *J Physiol Lond* 1969 204 461—474

Lloyd B B and R M Zacks The mechanical effic ency of treadmill runn ng against a horizontal impeding force *J Phys ol (Lond)* 1972 223 355—363

Thys H T Faraggiana and R Margaria Utilizat on of muscle elastic ty in exercise *J appl Phys ol* 1972 32 491—494

Whipp B J and K Wasserman Effic ency of muscular work *J appl Phys ol* 1968 26 644—648

Wise R M J F Rondinone and F N Briggs Effect of pCa on series-elast component of glycerinated skeletal muscle *Amer J Phys l* 1973 224 576—5 9

Zacks R M The mechanical eff ciencies of running and bicycl ng against a horizontal imped ng force *Int Z ang w Phys ol* 19 3 31 249—258

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From the Department of Physiology University of Oslo Norway

Pulmonary Responses to Intravascular Platelet Aggregation in the Cat

By

J VAAGE, G BO and J HOGNESTAD

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Abstract

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Platelet aggregation has been induced in cats by intravenous infusions of ADP collagen or platelet antiserum and the airway and pulmonary vascular responses subsequent to such infusions have been studied. Dynamic lung compliance (dyn C_L) pulmonary resistance (R_L) and the number of circulating platelets were followed together with pulmonary vascular resistance (PVR) Induction of platelet aggregation caused temporary and parallel increases in PVR and in R_L and a simultaneous decrease in dyn C_L The major part of these effects was apparently due to active smooth muscle constriction It could be shown that airway and vascular responses declined and finally disappeared in a characteristic way after repeated episodes of platelet aggregation. The total number of circulating platelets is apparently not the key factor in this connection. The way in which aggregation is induced as well as the functional state of the circulating platelets appear to be more important factors It may be that the platelet release recation is mandatory for the lung responses to intravascular platelet aggregation

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Experimental investigations indicate that blood platelet aggregates might be retained in the lungs and cause pulmonary dysfunction after pathophysiological events of various types such as non thoracic trauma (Robb 1963 Blaisdell Lim and Stallone 1970 Ljungquist Bergentz and Lewis 1971, Berman *et al* 1971) anaphylaxis (Robb 1967) hemorrhagic shock (Bo and Hognestad 1971), and endotoxin shock (Stein and Thomas 1967 Robb Margulis and Jabs 1972) Platelet aggregation is apparently also the all important change underlying the lung responses to experimentally induced pulmonary microembolization whether this be caused by blood clots (Thomas *et al* 1964) by barium sulphate (Bo Hognestad and Vaage 1974) or by air (Khan *et al* 1972)

Suddenly induced intravascular platelet aggregation appears to cause a rise in pulmonary vascular and airway resistance Some authors claim that this is mainly due to constriction of smooth muscles (Stein and Thomas 1967, Stein *et al* 1970 Swedenborg 1971 Radegran and McAslan 1972 Bo and Hognestad 1972) Others claim that physical obstruction of pulmonary vessels by platelet aggregates is of

major importance for the increased vascular resistance observed (Robb 1963 Hissen and Swank 1965 Swank 1968 Robb *et al* 1972 Schumacher and Classen 1972). Several investigators emphasize the importance of vasoactive substances released from the platelets (Stein and Thomas 1967 Swedenborg 1971 Bo and Hognestad 1972)

When intravascular platelet aggregation is repeatedly induced it has been shown that the pulmonary responses will gradually decline and finally be abolished (Bo and Hognestad 1972) During this change in the responses the animals remain in good general condition and they also retain a fairly high level of circulating platelets The decline in lung responses to repeated intravascular platelet aggregation could theoretically be due to either a decreasing sensitivity of the pulmonary smooth muscle cells or to qualitative and/or quantitative changes of the blood platelets We have previously suggested that the declining responses are due to functional changes in the circulating platelets as well as to a reduction in their number (Bo Hognestad and Vaage 1974) That platelets may become refractory to aggregating substances is known from *in vitro* studies (Rozenberg and Holmsen 1968) We felt that more information about the *in vivo* exhaustion of the blood platelets could yield valuable knowledge about the mechanisms behind the pulmonary responses to intravascular platelet aggregation The purpose of the present work therefore was to analyze this exhaustion phenomenon We also wanted to examine the nature of the airway responses and their relationship to the pulmonary vascular responses

Some of the results given have been presented previously in a short preliminary communication (Vaage Bo and Hognestad 1973)

Methods

Anesthesia and surgical procedures Cats weighing 2 5—4 5 kg were anesthetized by intraperitoneal injections of sodium pentobarbitone (Nembutal® Abbott) 30—40 mg/kg b wt The thorax was opened widely by a sternum splitting incision Polyethylene catheters were introduced into the femoral artery the pulmonary artery and the left atrium for recording of the femoral arterial pressure (P_{FA} with a Statham P23Db transduce) the pulmonary arterial pressure (P_{PA} with a Statham P23Db transducer) and the left atrial pressure (P_{LA} with a Statham P23De transducer) A flow probe was placed around the ascending aorta and cardiac output was recorded with a Nycotron square wave flowmeter (type 372 Nycotron A/S Norway) The pressure and flow transducers were connected to a polygraph (Grass Model 7 polygraph Grass Instrument Co Quincy Mass USA) A polyethylene catheter was inserted into the abdominal aorta through the femoral artery for arterial blood sampling Drugs and test substances were infused through a catheter into a femoral vein

Pulmonary vascular resistance PVR was calculated as the ratio ($\bar{P}_{PA}$—$\bar{P}_{LA}$)/mean aortic flow

Ventilation A tracheal cannula was inserted a muscle relaxant, Alloferin® (Hoffmann La Roche (0 1 ml/kg b wt)) was given and positive pressure ventilation initiated using a volume controlled respirator (The Ideal Respiration Pump C F Palmer Ltd London) The respiration frequency was 24 per min Before opening the thorax a positive end-expiratory pressure (1 5 cm H_2O) was applied to prevent lung collapse The tidal volume was adjusted so as to keep pH in arterial blood at about 7 40 at the start of the experiment Throughout the experimental period pH always changed less than 0 1 unit

Dynamic lung compliance and pulmonary resistance The tracheal air flow was measured by a screen pneumotachograph designed for small animals Hewlett Packard) and placed between the tracheal cannula and the respiration pump The pressure difference across screen was measured by a differential pressure transducer (Model ? 0 Hewlett P

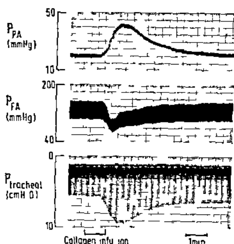


Fig 1

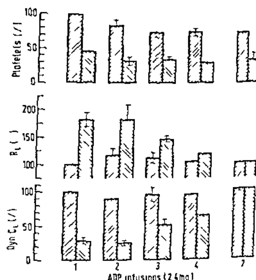


Fig 2

Fig 1 The effects on the airways and on the pulmonary circulation of intravascular platelet aggregation induced by intravenous administration of collagen in an open-chested cat The tracings show from above Pulmonary arterial pressure (P_{PA}) femoral arterial pressure (P_{FA}) and tracheal pressure

Fig 2 Changes in dynamic lung compliance (Dyn C_L) in airway resistance (R_L) and in circulating platelets upon repeated intravenous infusions of ADP in open chested cats The values represent mean ±S E in 4 animals Each pair of columns show values before and after infusion

calibrated so as to give air flow The airflow signal was electronically integrated (Respiratory Preamplifier Model 350—5000 B Hewlett Packard) to give the tidal volume The tracheal pressure which under open chest conditions equals the transpulmonary pressure was measured by a differential pressure transducer (Model 270 Hewlett Packard) Tracheal airflow tidal volume and tracheal pressure were continuously recorded during the experimental period on a Sanborn Polygraph (Model 320 Hewlett Packard) Dynamic lung compliance (dyn C_L) was calculated as the ratio of the tidal volume to the difference in transpulmonary pressure existing at the two situations of zero airflow within one respiratory cycle Pulmonary resistance (R_L) was calculated by dividing the difference in tracheal pressure at equal lung volumes within one respiratory cycle with the sum of the flow rates at these two points (Marshall 1965) Using this method we assumed a linear non elastic pressure flow curve and furthermore that the resistance during inspiration was the same as during expiration The resistance of the tracheal cannula was not subtracted

Infusions Repeated infusions over one min of 0 5 ml of either a 10 molar adenosine diphosphate (ADP) solution a collagen suspension prepared as described by *Holmsen* (1969) or platelet antiserum prepared as described by *Bø et al* (1974) were carried out

Ten min after each infusion when most of the response had vanished an inflation to a peak pressure of 20 cm H_2O was carried out A new infusion was then performed 10 min after this hyperinflation Measurements of dyn C_L and R_L were carried out within 1 min before each new infusion at peak response after the infusion and then at regular intervals until a new hyperinflation was performed

Thrombocyte counts in arterial blood samples were carried out according to the method of Brecher and Cronkite (1950) The number of circulating blood platelets was counted before each infusion at peak response and after the end of each response

pH measurements in arterial blood were carried out with a Radiometer pH meter 22

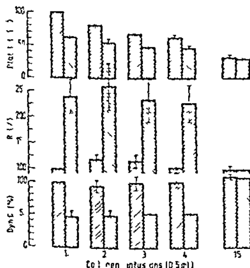


Fig 3

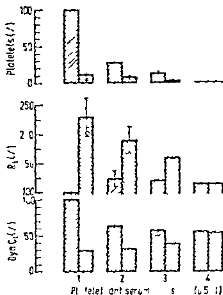


Fig 4

Fig 3 Changes in dynamic lung compliance (Dyn C_L) in airway resistance (R_L) and in circulating platelets upon repeated infusions of collagen in open-chested cats The values represent mean ±S.E. in 6 animals Each pair of columns shows values before and after infusion

Fig 4 Changes in dynamic lung compliance (Dyn C_L) in airway resistance (R_L) and in circulating platelets upon repeated intravenous infusions of platelet antiserum in open-chested cats The values represent mean ±S.E. in 7 animals. Each pair of columns shows values before and after infusion

Results

The pulmonary and systemic vascular responses to suddenly induced intravascular platelet aggregation were qualitatively the same whether ADP collagen or platelet antiserum was used as the aggregating substance and similar to the responses described by Bo and Hognestad (1972) Intravenous infusions of each of these substances caused a sharp rise in the pulmonary arterial pressure which reached a maximum about one min after the infusion was ended (Fig 1) The cardiac output did either fall or was unchanged whereas left atrial pressure remained constant Therefore pulmonary vascular resistance (PVR) increased A transient systemic hypotension was always seen as a part of the response (Fig 1)

Following an infusion the tracheal pressure did also rise (Fig 1) The time course of this rise was the same as that of the pulmonary arterial pressure The airway response was further analyzed by determining the change in pulmonary resistance (R_L) and dynamic lung compliance (dyn C_L) These changes as well as the number of circulating platelets after repeated infusions of ADP collagen or platelet antiserum are presented in Fig 2—4 For each type of substance the responses shown are based on experiments in 4 6 and 7 animals respectively For all three substanc

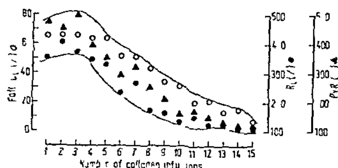


Fig 5 Effects of repeatedly induced intravascular platelet aggregation on dynamic lung compliance (C_L) on airway resistance (R_L) and on pulmonary vascular resistance (PVR) The results from one representative experiment where intravenous infusions of collagen were used to induce aggregation are shown

there was a tendency for the airway responses to decline and finally to disappear following repeated infusions The number of circulating platelets fell just after an infusion whereafter some increase was seen The post infusion fall in the number of circulating platelets was most marked and most permanent after platelet anti serum Subsequent to an infusion the values of dyn C_L and R_L did not reach their initial levels unless a deep lung inflation to an airway pressure of 20 cm H O was performed After platelet antiserum infusions however, even repeated hyperinfla tions were not able to restore dyn C_L and R_L to their initial levels

In each animal the first 2 infusions of ADP always gave identical airway responses After repeated infusions the lung responses were gradually reduced and they were completely abolished after 7 or 8 infusions (Fig 2) The number of circulating platelets returned incompletely after the first and second ADP infusion After subsequent ADP infusions reproducible and temporary reductions in the circulating platelets were observed with a post infusion recovery to about 70 per cent of the original value At the same time the various lung responses were gradually reduced When lung responses to ADP infusions could no more be elicited further ADP infusions did still markedly reduce the number of circulating platelets (Fig 2)

The initial infu ions of collagen gave airway responses similar to those caused by the ADP infusions (Fig 3) The number of circulating platelets showed a considerable reduction at the time when the airway response was at its maximum Afterwards a partial return of the circulating platelets was observed On successive infusions both the airway and the platelet response gradually diminished After about 15 infusions pulmonary responses could no more be elicited At this point the number of circulating platelets which amounted to about 30 per cent of the initial number showed no change following a new infusion of collagen (Fig 3)

The first infusion of platelet antiserum gave large lung responses and a large reduction in the number of circulating platelets (Fig 4) Only a poor reappearance of the platelets was seen (Fig 4) Even with a very low number of circulating platelets the second and third antiserum infusions did still give considerable lung responses No airway responses could however be elicited upon subsequent anti erum administrations A severe thrombocytopenia ($< 5000/mm^3$) was always present at this stage

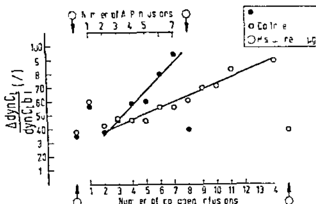


Fig 6 Effects of histamine (100 μg i v) on dynamic lung compliance early and late in a series of intravenous infusions of collagen and ADP Changes in dynamic lung compliance (Δ Dyn C_L) are given as per cent of the compliance level before each individual infusion of platelet aggregating substance (Dyn C_L b i) Results are given from two typical experiments with repeated administrations of ADP and collagen respectively

In 2 animals an infusion of collagen was carried out at a time when the pulmonary responses to ADP infusions had been exhausted The accompanying fall in dyn C_L (to 58 and 46 per cent of preinfusion level) was similar to the responses normally seen after the first infusion of collagen In 2 other animals ADP infusions were given at a time when there were no responses to collagen infusions No responses were then obtained with ADP either

When in 2 animals the responses to both ADP and collagen had been abolished an infusion of antiserum still elicited vigorous airway responses (dyn C_L dropped to 45 and 58 per cent of the preinfusion value) When on the other hand responses to antiserum could no more be obtained then both collagen and ADP also failed to cause any lung responses

The vascular responses to all these substances declined and disappeared simultaneously with the airway responses Fig 5 shows one typical example

In 5 animals the effect of an i v histamine infusion (100 μg) was observed initially and also when the responses to platelet aggregating substances had disappeared Two of the animals had received repeated collagen infusions and 3 of them repeated ADP infusions The fall in dyn C_L as well as the rise in PVR after histamine was the same in the two situations in all 5 expts (Fig 6)

Discussion

I v infusions of the platelet aggregating substances ADP collagen and platelet antiserum elicited pronounced pulmonary pressor responses confirming the observations from many previous investigations (Reeves *et al* 1967 Stein *et al* 1970 Hyman *et al* 1971 Bo and Hognestad 1972 Radegran and McAslan 1972) The pulmonary vessels and the airways were simultaneously affected suggesting some mutual relationship between these responses perhaps the influence of a common mediator The airway responses were evaluated as changes in dynamic lung compliance (dyn C_L) and pulmonary resistance (R_L) Induced platelet aggregation elicited a considerable decrease in dyn C_L and an incrase in R_L

Interpretation of airway responses

Changes in dyn C_L may be due to resistive changes, changes in functional residual volume (FRC) and changes *in specific compliance No determination of lung volumes* has been performed after experimental intravascular platelet aggregation, but Stein *et al* (1970) found reduced thoracic gas volumes after pulmonary embolization with autologous thromboemboli A part of the reduction in dyn C_L observed in our experiments is presumably caused by a reduced lung volume Nisell and Dubois (1954) have demonstrated a linear relationship between dyn C_L and FRC An active constriction of airway smooth muscles will precipitate airway narrowing and closure tending to reduce lung volumes and dyn C_L together We therefore believe FRC to be considerably reduced at the peak of the lung responses seen in the present series of experiments and thereby causing some reduction in dyn C_L

In normal subjects static compliance and dynamic compliance are similar However with increasing airway resistance especially in the small peripheral airways which contribute relatively little to total airway resistance (Macklem and Mead 1967) measurement of compliance will be dependent upon respiratory rate Thus resistive changes due to small airway constriction might influence dynamic lung compliance and explain some of the decrease in dyn C_L observed in the present experiments

No direct attempt was made to identify the site at which airway constriction took place in our experiments It appears likely however that larger airways as well as the more peripheral ones have been involved since dyn C_L decreased and R_L increased Hirose *et al* (1973) also suggested that airway constriction is located both in smaller and in larger airways after experimental pulmonary embolism with autologous thromboemboli in dogs During the response to intravascular platelet aggregation R_L was doubled a rise which is unlikely if larger airways did not constrict

Specific compliance may be reduced either due to a rise in alveolar surface tension or due to an increase in lung tissue elasticity It seems unrealistic to postulate any changes in lung surfactant rapid enough to occur during the brief response observed in our experiments Lung tissue elasticity might to some extent however be passively affected by pulmonary vascular congestion Hughes May and Widdicombe (1958) have demonstrated a 10 per cent reduction in C_L after a *doubling* of the pulmonary arterial pressure Bø Hauge and Waaler (1973) have demonstrated an inverse relationship between dyn C_L and the pulmonary blood volume Recently Vaage *et al* (1974) have demonstrated that pulmonary blood volume is increased at the peak of the pulmonary pressor response subsequent to a collagen infusion The blood *volume related reductions in dyn C_L are however* relatively *moderate* ones In the present experiments only a minor fraction of the observed large reduction in dyn C_L can be explained from hemodynamic changes in the lung *We therefore* suggest that the greater part of the compliance responses have been due to bronchiolar smooth muscle constriction

Simultaneously with the reductions in dyn C_L there was large rises in pulmonary resistance (R_L). The pulmonary resistance (R_L) measured in our investigations is the sum of airway resistance and viscous lung tissue resistance. Normally the tissue resistance comprises about 20 per cent of total R_L (Marshall and Dubois 1956 a). This fraction might increase but not enough to be an important component of total R_L (Marshall and Dubois 1956 b). For our purpose registrations of changes in R_L give information of changes in non-elastic airway resistance of a reasonable level of accuracy (Marshall 1965).

Non elastic airway resistance is dependent upon the airflow level, changes in lung volume and airway dimensions. No significant change in total airflow was observed in our experiments. Reduction in lung volume is also closely related to airway constriction and may account for some of the increase in R_L. It seems however that active constriction of conducting airways larger than 1 mm in diameter took place since such a constriction is the only factor which can explain the large rises in R_L observed in our experiments (Macklem and Mead 1967).

In summary platelet aggregation induces active constriction of airway smooth muscles which is suggested to change FRC and non-elastic resistance. These changes will together decrease dynamic lung compliance and increase pulmonary resistance.

Intrinsic mechanisms of the lung responses

What then is the origin of the bronchiolar constriction? A closer look at the response patterns after repeated infusions of different platelet aggregating substances seems to provide an answer to this question. It will be remembered that the pulmonary vascular responses were exhausted in parallel to the airway responses.

ADP infusions did initially cause marked pulmonary responses. However on repeated infusions there occurred a dissociation between the ability of ADP to cause lung responses and the ability of the substance to cause platelet aggregation. A considerable temporary reduction in the number of circulating blood platelets was still initiated by ADP when neither airway nor pulmonary vascular responses could be elicited. The number of platelets participating in an intravascular aggregation does therefore not by itself determine the magnitude of the pulmonary responses.

The airway responses to collagen did also diminish after repeated infusions. Here however there was a close correlation between the induced fall in the circulating platelets and the lung responses. Thus collagen induced airway responses as long as it induced measurable platelet aggregation (Fig. 3). Considerable lung responses could be elicited by collagen infusions even when the number of circulating platelets was fairly low. This pattern suggests a fundamental qualitative difference between the ADP and the collagen induced platelet aggregation.

Platelet antiserum induced a particularly marked thrombocytopenia and lung responses with few platelets returning to the circulation (Fig. 4). Even with a very low number of circulating platelets severe lung responses could be elicited by this substance. Also when airway responses to ADP and collagen had vanished completely platelet antiserum still provoked a considerable effect on the airways.

ADP stimulation induced a considerable platelet aggregation with subsequent reappearance of a high number of platelets. Platelet antiserum caused aggregation of most of the circulating platelets and only a minority of the aggregated platelets reappeared in the circulation. Collagen had effects intermediate to those of the other two agents. Consequently, there are different degrees of reversibility involved in the platelet aggregation induced by the three agents. There appears to be a causal connection between the degree of irreversible platelet aggregation on the one hand and the pulmonary responses elicited on the other, especially when few circulating platelets are available.

On repeated infusions of ADP the platelet aggregation and the secondary chain of events must apparently be changed in a qualitative way. Accordingly we suggest that both the functional state of the circulating platelets and the way in which platelet aggregation is induced are of importance for the pulmonary responses after intravascular platelet aggregation. Release of vasoactive substances might induce general smooth muscle constriction, vascular as well as bronchiolar. Platelet aggregation is known to elicit a platelet release reaction with the subsequent liberation of vasoactive substances like serotonin, histamine, adenine nucleotides and lysosomal enzymes (Holmsen, Day and Stormorken 1969). Recently prostaglandins released from platelets have also been suggested as possible mediators (Piper and Vaage 1974). In addition prostaglandins, slow reacting substance and histamine might also be released from the lung tissue proper (Piper and Vane 1969). Prostaglandins have been found to be released from lungs subsequent to experimental microembolization of lungs with artificial emboli (Piper and Vane 1971).

The dissociation between platelet aggregation and lung responses during ADP infusions and the fact that another platelet aggregating substance, collagen, which is a very powerful stimulus of platelet release, again could induce lung responses in the same animals, strongly suggests that the key event causing the lung responses during platelet aggregation is some special quality of platelet aggregation such as the release of vasoactive substances from the platelets. When the lung responses are exhausted, some qualitative alteration in platelet aggregation, probably the triggering of a release reaction, can definitely revive the lung responses. Thus a release of vasoactive substances from the lung tissue itself due to mechanical effects of the platelet aggregates cannot contribute essentially to the responses. Release from the lungs, if any, must be a secondary event to the release from platelets which probably is a condition sine qua non for the observed lung responses.

The fact that platelet aggregation and pulmonary pressor responses can be dissociated suggests that mechanical blockage of the pulmonary vessels does not play any dominant role for the pulmonary responses to *in vivo* aggregation of platelets. However, during active constriction of pulmonary vessels physical obstruction may be of some additional importance.

Neither collagen nor ADP induced any pulmonary pressor responses in animals made gravely thrombocytopenic by treatment with platelet antiserum. Therefore the lung responses normally seen following infusions of these drugs cannot be due to any direct effects on smooth muscle cells.

Histamine injections gave similar responses before and after the disappearance of the pulmonary responses to ADP and collagen Consequently the disappearance of the responses cannot be explained as the result of a general decrease in re activity of pulmonary smooth muscle cells

In conclusion the lung responses to intravascular platelet aggregation do in volve smooth muscle constriction This constriction depends on the number and the functional state of the circulating platelets as well as on the particular way in which aggregation is induced A key factor for the pulmonary pressor responses is probably the platelet release reaction

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References

BERMAN J R, M E SHULSON P PATTENGALE and S F SCHOENBACH Pulmonary microembolism after soft tissue injury in primates *Surg ry* 1971 *70* 246—53
BLAISDELL F W R. C LIM JR. and R. J STALLONE The mechanism of pulmonary damage following traumatic shock *Surg Gynec Obstet* 1970 *130* 15—2?
BRECHER G and E P CRONKITE Morphology and enumeration of human blood platelets *J appl Physiol* 1950 *3* 365—77
BO G and J HOGNESTAD Thrombocytes and pulmonary vascular resistance during hemor rhagic hypotension *Acta physiol scand* 1971 *8?* 218—28
BO G and J HOGNESTAD Effects on the pulmonary circulation of sud lenly induced intra vascular aggregation of blood platelets. *Acta physiol scand* 1972 *85* 523—31
BO G A HAUGE and B A WAALER The site of mechani ally induced changes in pulmonary blood volume and effects on lung compl ance *Acta physiol scand* 1973 *Suppl 396* p 100
BO G J HOGNESTAD and J VAAGE The role of blood platelets n pulmonary responses to microembolization with barium sulphate *Acta phys ol scand* 1974 *90* 244—51
HIROSE T T YASUTAKE A TARABEIH and M STEIN Location of airway constriction follow ing acute experimental pulmonary thromboembol sm *J appl Physiol* 19/3 *34* 431—3
HISSEN W and R L SWANK Screen f ltration pres ure and pulmonary hypertension *Amer J Physiol* 1965 *209* 715—2?
HOLMSEN H *Adenine nucleotide metabolism of blood platelets* Thesis Uni ersitetsforlaget Oslo 1969
HOLMSEN H H J DAY and H STORMORKEN The blod platelet release reaction *Scand J Ha mat* 1969 suppl 8
HUGHES R A J MAY and J G WIDDICOMBE The effect of pulmonary congestion and oedema on lung compl ance *J Physiol* Lond) 1958 *14?* 306—13
HYMAN A L W C WOOLVERTON D G PENNINGTON and W E JAQUES Pulmonary vascular responses to adenos ne diphosphate *J Pharmacol exp The* 1971 *178* 549—61
KHAN M A I ALKALAY S SUETSUGU and M STEIN Acute changes in lung mechanics fol lowing pulmonary emboli of arious gases in dogs *J appl Phys ol* 197? *33* 774—77
LJUNGQUIST U S E BERGENTZ and D H LEWIS The distribution of platelets f brin and erythrocytes in arious o gans following experim ntal trauma *Eu op Surg Res* 1971 *3* 93—300
MACKLEM P T and J MEAD Resistance of central and peripheral airw a s measured by a retrograde catheter *J appl Physiol* 196/ *22* 395—401
MARSHALL R Objective tests of resp ratory mechan cs *Handbook of Physiology* section 3 Resp ration of II American Physiological Soc et Wash ngton D C 1965
MARSHALL R and A B DUBOIS The measurement of iscous resistance of the lung ti sues in normal man *Clin Sc* 1956 a *15* 161—70
MARSHALL R and A B DUBOIS The is ous resistance of the lung tissue in pat ents with pulm onary disease *Cl n Sc* 1956 b *15* 473—83
NI ELL O I and A B DUBOIS Relationsh p between compl ance and FRC of the lungs in cats and measur ment of re istance to breathing *Ame J Physiol* 1954 *1/8* 206—10
PIPER P J and J VAAGE Platelet aggregation pulmonary microembolism and the release prostagland ns *Mic ovasc Res* 19/4 In press

PIPER P J and J R. VANE Release of additional factors in anaphylaxis and its antagonism by anti inflammatory drugs *Nature* (Lond) 1969 *223* 29—37
PIPER P J and J R. VANE The release of prostaglandins from lungs and other tissues. *Ann N Y Acad Sci* 1971 *180* 363—80
REEVES J T P JOKL, J MERIDA and J E. LEATHERS Pulmonary vascular obstruction following administration of high-energy nucleotides *J appl Physiol* 1967 22 475—79
ROBB H The role of microembolism in the production of irreversible shock. *Ann Surg* 1963 *158* 685—92
ROBB H Observations on dynamics of microcirculation during experimental anaphylaxis *Ann Allergy* 1967 25 78—87
ROBB H R. MARGULIS and C JABS Role of pulmonary microembolism in the hemodynamics of endotoxin shock. *Surg Gynec Obstet* 1972 *135* 777—83
ROZENBERG M C and H. HOLMSEN Adenine nucleotide metabolism of blood platelets. IV Platelet aggregation reseponse to exogenous ATP and ADP *Biochim biophys Acta* (Amst.) 1968 *157* 280—88
RADEGRAN K. and C. MCASLAN Circulatory and ventilatory effects of induced platelet aggregation and their inhibition by acetylsalicylic acid *Acta anaesth scand* 1972 *16* 76—84
SCHUMACHER K A and H. C CLASSEN Platelet aggregation and increased pulmonary vascular resistance in cats induced by DAS and ADP *Naunyn-Schmiedeberg's Arch. exp Path Pharmacol* 1972 *275* 373—81
STEIN M and D P THOMAS Role of platelets in the acute pulmonary response to endotoxin. *J appl Physiol* 1967 *23* 47—52
STEIN M T HIROSE T YASUTAKE and M A KAHN The effects of platelet amines on airway function in *Airway dynamics* ed. A Bouhuys pp 283—95 Charles C Thomas, Springfield Ill U.S.A. 1970
SWANK R L Platelet aggregation its role and cause in surgical shock. *J Trauma* 1968 *8* 872 – 9
SWEDENBORG J Thrombin induced vasoconstriction in the pulmonary circulation *Scand J clin Lab Invest* 1971 27 321—29
THOMAS D M STEIN G TANABE V REGE and S WESSLER, Mechanism of bronchoconstriction produced by thromboemboli in dogs *Amer J Physiol* 1964 206 1207—1212
VAAGE J G Bo and J HOGNESTAD Pulmonary responses to repeatedly induced intravascular aggregation of blood platelets *Microvasc Res* 1973 6 257
VAAGE J P AARSETH I ENGE and J HOGNESTAD Changes in pulmonary vascular volumes after induced intravascular aggregation of blood platelets *Acta physiol. scand* 1974 91 84—94

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From the Psychological Laboratories University of Stockholm Sweden

Catecholamine Output of Males and Females over a One-Year Period

By

GUNN JOHANSSON and BIRGITTA POST

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Abstract

JOHANSSON, G and B POST *Catecholamine output of males and females over a one year period* Acta physiol scand 1974 92 557—565

Urine samples were collected under constant conditions once every fourth week over 1 year The subjects 13 male and 11 female office employees and workers in industry participated in the study during their ordinary daily work routine Large interindividual differences in the rate of catecholamine excretion were obtained whereas most subject showed a high intra individual constancy of excretion Neither variations over the year nor differences between men and women in the excretion of adrenaline or noradrenaline were statistically significant. However the women differed from the men in that their adrenaline excretion was not significantly higher in a test session than during daily work routine

The excretion of catecholamines especially that of adrenaline has been shown to be a sensitive indicator of arousal in a great number of studies of the effects of various psychosocial stressors (see review by Frankenhaeuser 1971) Thus the output of adrenaline and noradrenaline is increased not only in response to threatening physical stimuli—the fight or flight reaction described by Cannon—but also in situations involving for example mental work under time pressure (Frankenhaeuser and Patkai 1965) and under anticipation and uncertainty (Frankenhauser and Rissler 1970)

Circulating adrenaline has been shown to be of great significance for the regulation of human behavior Adrenaline excretion for instance is positively related to behavioral efficiency (*e g* Patkai 1970)

One of many important physiological functions of circulating catecholamines is in the complex mechanism of centrally mediated temperature regulation and acclimation (Leduc 1961)

In investigations which focus on relationships between the psychosocial environment catecholamine secretion and human behavior extreme temperatures are avoided However in a temperature climate humans are constantly exposed to slow seasonal climatic variations requiring gradual adjustment It is also possible that

An intelligence test and a personality inventory were administered The subjects also filled in a questionnaire concerning their age previous illnesses frequency of insomnia and a fe other data. Before the test session each subject emptied his bladder and after the session urine samples were collected Each subject was paid 25 Swedish Kr (about 6 U S $) for participation in the test session

Dependent variables

Catecholamine excretion The volume of each urine sample was immediately measured and its pH was adjusted to about 3 by the addition of 2 N HCl Samples were then stored at −18 C for a year until the last set had been collected Each subject's urine samples were then analyzed simultaneously using the fluorimetric method of Euler and Lishajko (1961)

Intelligence and personality data The psychometric methods used were the Eysenck Personality Inventory (Eysenck and Eysenck 1964*) and a factor type intelligence test named the test (Harnqvist 1961) In both cases raw scores were calculated

Other variables

Data of body weight amount of sleep, and a few other variables which could not be strictly controlled but which might affect the results were collected as well as data on air pressure and outdoor temperature which were used as indicators of seasonal changes

Body weight Subjects were weighed on each occasion of urine collection

Consumption of caffeine and nicotine The number of cigarettes pipes cups of coffee and etc consumed by the subject during the two hour periods was noted on each occasion

Subjective data and amount of sleep On each regular morning of investigation subjects gave estimates of fatigue and work load They were instructed to state whether they felt just as tired as they usually are in the morning at the particular time of the year or whether they were more or less tired than that Similarly they stated whether their work load was higher or lower than usual or if it was ordinary for the time of the year Finally the subjects noted on the questionnaire how many hours they had slept during the preceding night

Air pressure and outdoor temperature Data on temperature and air pressure in a close by town were obtained from the Monthly and Yearly Summary of Weather and Water Supply in Sweden (Vol 51—52 1969—1970) issued by the Swedish Meteorological and Hydrological Institute These data showed almost perfect agreement with measurements of temperature and pressure taken by the experimenters in the town of the investigation

Results

Some observations were lost due to illness or temporary change of work shifts The number of observations for single occasions varied between 16 and 24 and for single subjects between 8 and 13

Intra and interindividual comparisons

Fig 1 shows means and standard errors of adrenaline and noradrenaline excretion for single subjects over the year The parameters have been calculated on catecholamine measures after correction for body weight The means for noradrenaline excretion range from 0 23 to 0 54 ng/min/kg in women and from 0 21 to 0 71 ng/min/kg in men The maximum individual standard error was only ±0 07 ng/min/kg the same for both sexes The average adrenaline excretion varied between 0 03 and 0 11 ng/min/kg among female subjects and between 0 03 and 0 19 ng/min/kg among the males The maximum standard errors were ±0 03 and ±0 07 ng/min/kg respectively Thus the intraindividual consistency was high as compared with the interindividual differences a tendency which was stronger for noradrenaline than for adrenaline excretion

* Translated into Swedish and modified by Bederoff Peterson Jagtoft and Asur m Scandin Test Corporation 1971

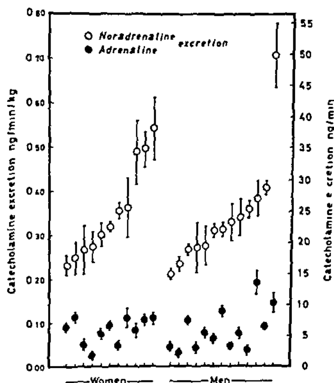


Fig 1 Individual means and standard errors for adrenaline and noradrenaline excretion in men and women Measurements were obtained at 4 week intervals during one year and have been corrected for body weight The right hand scale gives corresponding values in ng/min for a fictive 70 kg person

Sex differences in catecholamine excretion will be analyzed further below (p 56?)

Seasonal comparisons

Since large interindividual differences in catecholamine excretion were found, a loss of observations on subjects with extreme levels might result in systematic errors Thus for the purpose of comparisons over the year catecholamine excretion measures were transformed for each subject separately into standard scores of zero mean and unit standard deviation

Fig 2 shows the variations in adrenaline and noradrenaline excretion over the year of investigation along with data on temperature and air pressure Both adrenaline and noradrenaline excretion are fairly stable and in only one instance does the mean excretion of either hormone exceed ±0 5 standard deviation units a value which may be compared to ±1 96 the 5 % significance interval Adrenaline excretion was slightly higher in the spring and the fall than during the rest of the year and the noradrenaline excretion was highest in December and February

The transformed data were submitted to a two way analysis of variance allowing uneven cell numbers A summary of the results is presented in Table I which shows that no statistically significant differences were found either between sexes or between occasions of urine collection Interaction effects were also non significant

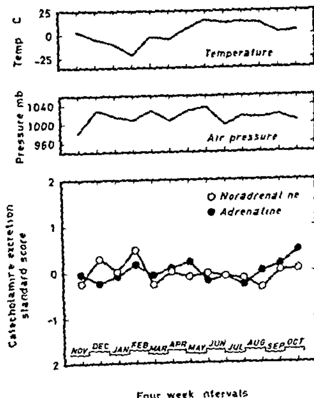


Fig 2 Mean adrenaline and noradrenaline excretion in a group of 11 women and 13 men at four week intervals over a year The measures have been transformed for each subject separately into standard scores of zero mean and unit standard deviation The upper diagram shows air pressure and outdoor temperature on the days of urine sampling

The consumption of nicotine and caffeine during the two hours of investigation was very stable over the year The average consumption was 2 cigarettes and 1 5 cups of coffee The average amount of sleep during the night preceding urine sampling was 6 6 h

Daily work routine vs test session

For the comparison of catecholamine output between the test session and daily work routine raw data with no transformation or correction for body weight were

TABLE I Summary of analyses of variance of adrenaline and noradrenaline excretion of 11 women and 13 men for whom urine samples were collected on 13 occasions over one year (Measures of excretion have been transformed into standard scores with M = 0 00 and S D = 1 00 for each subject separately)

Source of variance	df	Adrenaline MS	F	Noradrenaline MS	F
Sex	1	0 03	0 075 n s	0 18	0 18 n s
Time	12	0 68	0 6 6 n	1 34	1 364 n s
Sex x time	14	1 40	1 400 n	1 0	1 087 n s
Within cell	230	1 00		0 98	
Total	255				

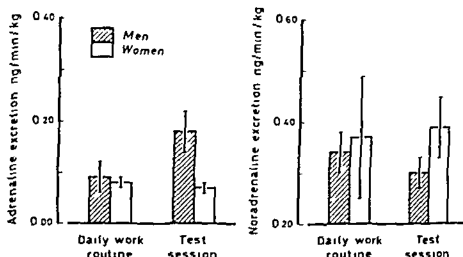


Fig 3 Means and standard errors of adrenaline excretion (left hand diagram) and noradrenaline excretion (right hand diagram) expressed in relation to body weight of 11 women and 13 men respectively during daily work activity and a mental test session

used Each subject's average excretion during daily routine activity over the year was computed and compared with the catecholamine excretion during the test session It should be kept in mind that the type of activity was not the only difference between the two conditions since subjects were allowed to smoke in one condition but not in the other It was found that for the entire group of subjects the difference between conditions was non significant for adrenaline as well as for noradrenaline (Table II) However when data for the two sexes were treated separately the group of males was shown to have significantly elevated adrenaline levels during the test session as compared with daily work routine ($t = 2\ 67$ $p < 0\ 05$ $df = 11$) Neither sex group showed any significant change of noradrenaline output

Sex differences

In order to find out if the sex groups differed in their absolute catecholamine levels as well as in their catecholamine reactivity weight corrected measures of adrenaline

TABLE II Adrenaline and noradrenaline excretion in the total group of subjects during daily work routine and during a performance test session The table shows means and standard errors as well as t values for the difference between conditions

Variable	Daily routine activity M	S E	Test session M	S E	t
Adrenaline excr ng/min	[illegible]	0 5	8 5	1 5	−1 768 n s
Noradrenaline excr ng/min	23 3	1 7	23 8	1 9	−0 196 n s

TABLE III IQ scores and personality data for men and women The table shows means and standard errors as well as t values for differences between the sexes (df = 21)

Variable	Men M	S E	Women M	S E	t
IQ test, sum of raw scores	139 9	8 7	150 4	9 4	−0 81 n s
EPI extraversion score	12 4	0 7	11 9	1 2	0 38 n s
EPI neuroticism score	6 4	1 2	10 4	1 6	−1 99 n s

and noradrenaline were used The results of the comparisons are shown in Fig 3 There was no significant difference in adrenaline excretion between sexes when the subjects carried out their ordinary jobs In the test session however the men excreted significantly more adrenaline than the women ($t = 2\ 50$ $p < 0\ 05$ $df = 21$)

Noradrenaline excretion did not differ significantly between sexes in either of the two conditions

Men and women were compared in terms of psychological variables Table III shows that the differences were small and statistically non significant

Discussion

This study has provided information on the urinary excretion of catecholamines in humans over an extended time period under everyday conditions This kind of data provides an important complement to the data collected in laboratory experiments which usually refer to brief time periods Large interindividual differences were found whereas the intraindividual variations were in general rather small These results are in agreement with those of Patkai *et al* (1974) who studied women during two menstrual cycles

The results of this study indicate that the urinary excretion of catecholamines in humans remains stable over seasons in a temperate climate Thus the excretion of adrenaline and noradrenaline by men and women carrying out their ordinary work under constant conditions did not differ significantly between seasons

In two earlier investigations of humans higher levels of catecholamines were found in the autumn and winter than in the spring (Feller and Hale 1964 Johansson *et al* 1969) However since in the earlier studies different subjects were studied during the different seasons interindividual differences in habitual levels of catecholamine excretion may have caused the differences In the present study only a minor non significant elevation during the winter was found for noradrenaline

The excretion of adrenaline and noradrenaline during daily work routine was also compared to those of a test session In agreement with many studies of performance stress (see *e g* Frankenhaeuser 1971) the adrenaline levels in males were significantly higher in the test session than during daily routine activity The women—

however, excreted the same amount of adrenaline in both conditions. In this context it should be pointed out that subjects were allowed to drink coffee and to smoke during the daily work routine but not in the test session and that the women tended to smoke slightly more than the men To check the possible influence of these factors a subgroup of three female and four male non smokers who drank no coffee was studied separately In this group all men excreted more adrenaline than any of the women in the test session whereas there was a total overlap in adrenaline levels between the sexes during daily work activity

This sex difference in adrenaline excretion during mental effort should be interpreted with caution but it is particularly interesting since a study of 12 year old school children in a mentally passive and a mentally active school situation (Johansson 1972) showed a similar sex difference in adrenaline reactivity In most of the research concerning catecholamine reactivity in stress male subjects only have been studied However in a recent study on dysmenorrhea Patkai and Petersson (personal communication) obtained data on stress reactivity in females Their subjects were exposed to a complex reaction time test and it was shown that the adrenaline excretion of a control group of women with normal menstrual complaints remained unaffected during the test as compared with a basal condition The same test has in previous studies caused a marked increase of adrenaline levels in men (Johansson and Frankenhaeuser 1973)

In a few investigations both sexes have been studied and higher hormone levels have been found in males than in females (*e g* Lambert *et al* 1969) a fact that has been explained in terms of differences in body weight The present data support the assumption that correction for body weight eliminates the sex differences in hormone excretion during conditions of relaxation but that males in situations of mental work tend to react with higher adrenaline output than females It is interesting to speculate about possible explanations to this difference Close at hand is the suggestion that sex role socialization may create different patterns of competition in males and females so that a test situation actually has different significance to males and females (Johansson 1973) It could be argued that the higher levels of the males reflect a more intense involvement in the work However the performance scores of the present study did not differ between sexes and thus do not support such a conclusion It is also possible that men and women differ with respect to endocrine thresholds so that the subjective intensity of stimulation required for adrenal medullary response may be different for the sexe Further investigations will be needed in order to determine how general the sex difference is

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References

DAVIES T W A seasonal change in the diurnal rhythm of the excretion of 3 m thoxy hydroxymandelic acid in man *Quart J exp Physiol* 19/0 *55* 122—128
EULER C v The physiology and pharmacology of temperature regulation with particular reference to the chemical mediators In *Biochemical and Neurophysiolog l Cor ela ion of Centrally Acting Drugs* Oxford Pergamon Press 1964
EULER U S v and F LISHAJKO Improved technique for the fluorimetric est n ation of catecholamines *Acta physiol scand* 1961 *51* 348—355
EYSENCK H J and S B G EYSENCK *Manual of the Eysenck Personal ty Inv nt ry* London University of London Press 1964
FELLER R. P and H B HALE Human urinary catecholamines in relation to climate *J appl Physiol* 1964 *19* 37—39
FRANKENHAEUSER M Behavior and circulating catecholamines *B ain Res* 1971 *31* 41—262
FRANKENHAEUSER M and P PATKAI Interindividual differences in catecholamine excretion during stress *Scand J Psychol* 1965 6 117—123
FRANKENHAEUSER M and A RISSLER Catecholamine output during relaxation and anticipation *Percept Mot Skills* 1970 *30* 745—746
HARNOVIST K *Manual till F provet [Manual of the F test]* Scand Test Corp 1961
JOHANSSON G Sex differences in the catecholamine output of children *Acta physiol scand* 1972 *85* 569—572
JOHANSSON G Activation adjustment and sympathetic adrenal medullary activity Field and laboratory studies of adults and children *Rep Psychol Lab* Univ Stockholm. 1973 Suppl 21
JOHANSSON G and M FRANKENHAEUSER Temporal factors in sympatho-adrenomedullary activity following acute behavioral activation *Biol Psychol* 1973 *1* 63—73
JOHANSSON G M FRANKENHAEUSER and W W LAMBERT Note on seasonal variations in catecholamine output *Percept Mot Skills* 1969 *28* 677—678
LAMBERT W W G JOHANSSON M FRANKENHAEUSER and I KLACKENBERG-LARSSON Catecholamine excretion in young chi dren and their parents as related to behavior *Scand J Psychol* 1969 *10* 306—318
LEDUC J Catecholamine product on and release in exposure and accl mation to cold *A ta physiol scand* 1961 *53* Suppl 183
PATKAI P Relation between catecholamine release and psycholog cal functions *Rep Psychol Lab* Univ Stockholm 1970 Suppl 2
PATKAI P G JOHANSSON and B POST Variations in physiological functions during the menstrual cycle *Psychosomat Med* 1974 (In press)

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From the Institute of Medical Biology Sect of Physiology, University of Tromsø and Institute of Aviation Medicine Royal Norwegian Air Force Blindern Oslo Norway

Arterial Hypoxia and the Diving Responses of Ducks

By

ARNOLDUS SCHYTTE BLIX[1] and TROND BERG

After a short latent period (1—10 s) ducks respond to head immersion with a progressively increasing cardiac retardation which is usually fully developed in 30—40 s (*e g* Fig 1) At this stage the arterial PO_2 has decreased to about 45 mm Hg Jones and Purves (1970) have reported that denervation of the arterial chemoreceptors will abolish most of the diving bradycardia Moreover Andersen and Blix (1974) and Blix *et al* (1974) demonstrated that the diving bradycardia of ducks also depends upon the development of peripheral vasoconstriction This might imply that the peripheral vasoconstriction and thereby the bradycardia of ducks as opposed to in seals develops gradually in response to increasing chemoreceptor stimulation

The present study was undertaken in order to test if arterial hypoxia without hypercapnia affects the development of diving bradycardia in ducks

The experiments were carried out in a 16 m³ low pressure chamber within which both the duck and the experimentator (the latter with oxygen equipment) could be exposed to simulated altitudes of up to 12 000 m By decreasing the pressure inside the chamber the arterial PO_2 of the animal will decrease *without* an increase of arterial PCO_2 although the duck is freely breathing

6 domestic ducks weighing about 3 kg were used The animal were restrained ventral side down and the brachial arteries cannulated with polyethylene catheters (PP 100) during local anesthesia Central arterial blood pressure was measured through one of the catheters by u e of a Statham transducer (*P23H*) connected to a Beckman RS polygraph The other arterial catheter was used for *sampling* of blood The arterial PO_2 PCO_2 and pH were determined by use of Radiometer electrodes The ventilation frequency was recorded by a thermocouple placed *in the* nare opening and connected to the polygraph In all experiments *diving* was simulated by head immersion in water of about 25° C

The ducks were submerged under 3 different situations First at sea level where the arterial PO_2 was determined at the moment of fully developed bradycardia Second, at an altitude (6000 m) which resulted in a pre dive PO_2 slightly below the value obtained at the time of fully developed bradycardia during the sea level

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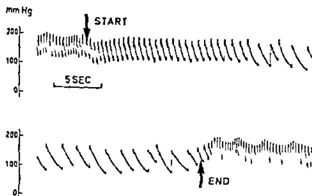


Fig 1 The normal cardiovascular response to diving in the duck The figure includes arterial blood pressure recordings from the beginning (upper tracing) and the end (lower tracing) of a dive lasting for 40 s

dive Third at the same altitude (6000 m) but with the chamber enriched in O sufficient to give the duck the same pre dive arterial PO_2 as at sea level

The normal cardiovascular response to diving in the duck is shown in Fig 1 The arterial PO fell during the initial 15 s period from 91 9±4 4 (S D) to as low as 40 mm Hg Following this initial drop the arterial PO_2 decreased gradually by 3—5 mm Hg for each subsequent 30 s period

Exposure *of the duck to an altitude of 6000 m (ambient PO = 73 mm Hg)* resulted in a steady state arterial PO of 33 0±3 3 (S D) mm Hg When submerged at this altitude the duck responded with fully developed bradycardia after a delay of about 3 s This typical response (four ducks) is shown in Fig 2 while two ducks revealed a slight but gradual blood pressure increase after the same prompt onset of fully developed bradycardia

Diving at the same altitude (6000 m) after adjustment of the arterial PO to sea level value revealed a cardiovascular response just like that obtained at sea level

Upon ascent to 6000 m the ventilation frequency increased from 15 to 19 breath pr min on average while the heart rate increased from 180 to 215 beats pr min

At sea level the arterial PCO and pH were 29 5±3 8 (S D) and 7 48±0 04 (S D) respectively while *the corresponding values at altitude* (6000 m) were 24 2 ±3 5 (S D) and 7 58±0 03 (S D)

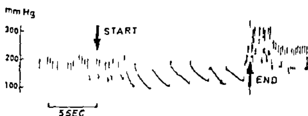


Fig 2 The arterial blood pressure response to head immersion in a duck which entered the dive at an altitude of 6000 m

In our opinion, neither the increased arterial pH, nor the slightly decreased arterial PCO_2 is likely to produce the prompt onset of the bradycardia in the high altitude dive It follows that the gradually increasing arterial hypoxia, appears to be an important factor in the regulation of the development of the cardiovascular responses (*e g* vasoconstriction and bradycardia) to normal dives in the duck

We wish to express our appreciation to Mr Arne Rettedal for his ample assistance during the experiments and to Prof J B Steen for his valuable criticism and suggestions during the preparation of the manuscript

References

ANDERSEN H T and A S BLIX Pharmacological exposure of components in the autonomic control of the diving reflex *Acta physiol scand* 1974 *90* 381—386

BLIX A S EVA L GAUTVIK and H REFSUM Aspects of the relative roles of peripheral vasoconstriction and vagal bradycardia in the establishment of the diving reflex in ducks. *Acta physiol scand* 1974 *90* 289—296

JONES D R and M J PURVES The carotid body in the duck and the consequences of its denervation upon the cardiac responses to immersion *J Physiol* (Lond) *1970 211* 279—294

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From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

Effect of Acidification of Totally Denervated Pouches of the Duodenal Bulb on Gastric Acid Secretion in Dog

By

GORAN NILSSON

In previous studies evidences have accumulated that acidification of isolated pouches of the proximal portion of the duodenum i e the duodenal bulb evokes inhibition of gastric acid secretion by releasing a humoral agent (Nilsson 1969 a) If so inhibition of acid secretion should also appear at acidification of bulbar pouches completely deprived of their nervous supply The present study was undertaken to compare the effect of bulbar acidification on gastric acid secretion before and after partial respectively total denervation of the bulbar pouches

2 dogs weighing 16—20 kg were provided with fully innervated pouches of the fundic stomach (Pavlov type) and of the proximal portion of the duodenum corresponding the duodenal bulb (Andersson and Uvnas 1961) In a subsequent operation the pylorus was divided and pyloric portion of the bulbar pouch was brought to the abdominal wall and peritonealized In a final operation all original vascular and nervous supply to the bulbar pouch and to the ileal loop connecting the bulbar pouch with the exterior of the abdominal wall was removed so that the bulbar pouch now exclusively received its vascular supply from the abdominal wall Between operations and before experiments were started the dogs were allowed 3—5 weeks for recovery

Experiments were started in the morning after 16—18 hours of fasting Basal acid output was recorded for 1 hour During the basal period and for the following 5 hours acid secretion was collected in 15 min samples The volume was measured and the acidity determined with 0 01 N NaOH and with phenolphthalein as indicator

Acid secretion was stimulated by pentagastrin (ICI 50123) Before the experimental series was started, acid output to graded doses of pentagastrin was determined in each dog From those experiments a dose of pentagastrin (40 μg/h) was selected that evoked secretory rates from the Pavlov pouches corresponding 20—40 % of their maximal secretory response to pentagastrin The pentagastrin was given in 0 9 % NaCl as a continuous intravenous infusion for 5 hours and at a rate of 10 ml per hour by a calibrated peristaltic pump (Harvard aparatus Co Dover Mass) At the beginning of the intravenous infusion perfusion of the bulbar pouches with 0 9 % of NaCl was started After 2 1/2 hours saline was substituted for 0 1 N HCl. After one hour of bulbar acidification saline was perfused through the bulbar pouches for another 1 1/2 hours Further details of the bulbar perfusion procedure have been given elsewhere (Andersson Nilsson and Uvnas 1967)

In a series of studies the characteristics of the bulbar mechanism that inhibits gastric acid secretion have been investigated Those studies show that acidification of isolated bulbar pouches effectively inhibits acid secretion from both vagally innervated (Andersson and Uvnas 1961 Andersson Nilsson and Uvnas 1967 Nilsson 1969 b Andersson and Nilsson 1969 and Nilsson 1969 c Nilsson and Rune 1971 Andersson and Sjodin 1972) and vagally denervated (Andersson *et al* 1967 An

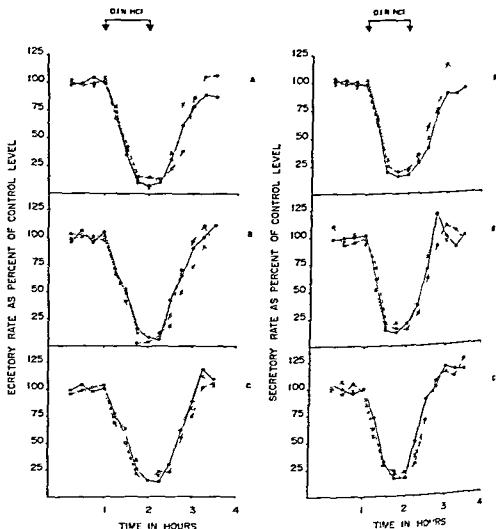


Fig 1

Fig 1 Effect of bulbar perfusion with 0 1 N HCl on secretory responses to gastrin in 2 Pavlov pouch dogs with fully innervated bulbar pouches (A) after transection of the pylorus (B) and with totally denervated pouches of the duodenal bulb (C) Each curve represents one experiment.

dersson and Nilsson 1969) gastric pouches Duodenal inhibitory experiments have also been performed on dogs with gastric pouches deprived of both parasympathetic and sympathetic nervous supply Andersson 1963) In that study acid was not perfused through isolated pouches of the duodenal bulb but introduced into the excluded duodenum through a duodenal cannula By this technique attempts were made to acidify the proximal portion of the duodenum Acid leaking into the lower portion of the duodenum most likely became neutralized by bile and pancreatic juice entering the duodenum The observed inhibition was therefore probably due to activation of the bulbar inhibitory mechanism and not to release of secretin that

may be responsible for inhibition of acid secretion at acidification of lower portic of the duodenum (Konturek and Grossman 1965) Although the related re u (Andersson 1963) strongly indicated that duodenal acidification cau es secreto y inhibition by releasing a humoral agent the author could not exclud that acidifica tion of the innervated duodenum produced reflex liberation of adrenalin that re duced the acid secretion from the denervated gastric pouch

The present results (Fig 1) confirm previous observations that acidification of fully innervated bulbar pouches effectively inhibits gastric acid response to penta gastrin (Nilsson and Rune 1971) When experiments were repeated follo ing tran section of the pylorus inhibition remained unchanged indicating that intramural reflex mechanisms between the duodenum and the stomach play a minor role or are not involved in bulbar inhibition of acid ecretion Similar results have also been obtained in studies using test meal sham feeding or insulin hypoglycemia as secretory stimulants (Andersson and Sjodin 1972) Also when the fully denervated bulbar pouches were acidified inhibition occurred and this reduction of acid output did not differ from inhibition obtained in fully innervated gastric pouches The present results therefore prove that reduction of the intrabulbar pH evokes release of a humoral agent that inhibits gastric acid secretion The name bulbogastrone has been suggested for this inhibitory principle (Anders on Nilsson Sjodin and Uvnas 1973)

This study was supported by research grants from the Swedish Medical Research Council (project No 3571) The author is grateful to Miss Gunilla Thulin Mrs Elisabeth Tiren Mrs Inga Lill Glans and Miss Sara Belchato ska for techn cal and secretarial assistance

References

ANDERSSON S Inhibition of gastric secret on by duodenal acidificat on before and after sympathetic denervation of H id nhain pouches *Gastroent rology* 1963 *45* 752—755

ANDERSSON S and G NILSSON pH-dependence of the mechanism n the duodenal bulb in h bi ing gastric acid responses to exogenous gastrin *Acta physiol scand* 1969 *76* 187—190

ANDERSSON S and L SJODIN Inhib tion of gastric secret on by ac dif cation of the duodenal bulb before and after tran ection of the pylorus *Scand J Gast oent* 1972 *7* 151—156

ANDERSSON S and B UVNAS Inh bition of postprandial gastric secretion in Pavlov pouches by instillation of hydrochloric acid into the duodenal bulb *Gastroenterology* 1961 *41* 486—490

ANDERSSON S G NILSSO and B UVNAS Effect of ac d in proximal and distal duodenal pouches on gastric sec etory respon es to gastrin and histam ne *Acta phy iol scand* 1967 *71* 368—378

ANDERSSON S G NILSSON L SJODIN and B UVNAS Mechanism of duodenal inhibition of gastric ac d sec etion *Nob l Symposium VII* F ont rs in gastrointestinal hormone research Almqv st & Wiksell Stockholm 1973 273—238

KONTUREK S and M I GROSSMAN Localization of the mechanism for inhibition of gastr c secretion by a d n testin *Ga t oent rology* 1965 *49* 14—78

NILSSON G *Studie of the mechanism by wh ch ac d n the duodenal bulb inhib t gast c a d se t on* Thes s Stockholm 1969 a

NILSSON G Eff ct of ac d in the duodenal bulb on gastric secretory re ponses to insulin hypogl cemia *A ta phys ol s and* 1969 b *75* 416—483

NILSSON G Effect of acid n the duodenal bulb on gastric secretory responses to sham feed ing *A t phy ol cand* 1969 *77* 308—315

NILSSON G Eff ct of acid in the duodenal bulb on plasma gastrin and gastric acid re ponses to ham feeding *Acta physi l s a d* To be published

NILSSON G and S RUNE Failu e of high pCO_2 in the duodenal bulb to inhib t gastri a secreti n *A ta phys ol scand* 1971 *81* 324—354

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From the Department of Physiology and Biophysics University of Lund Sweden

Beta Adrenergic Micro-Vascular Dilatation Evoked by Sympathetic Stimulation

by

JAN LUNDVALL and JOHANNES JARHULT

Viveros Garlick and Renkin (1968) reported that the vascular response evoked by activation of the adrenergic sympathetic fibres to the dog skeletal muscle includes a β adrenergic dilator component This effect normally concealed by simultaneous α adrenergic constriction was revealed in the presence of the selective α blocking agent Dibozane as a moderate dilatation of the resistance vessels there was some indication of an influence on precapillary sphincters as well Further evidence for a sympathetic β adrenergic dilator effect in skeletal muscle was recently given by Tuttle and Moe (1973) from cat experiments The physiological significance of this vasodilatator mechanism is however so far unknown

The present study shows that neurogenic β dilatation in skeletal muscle mainly is confined to resistance vessels of small diameter and to precapillary sphincters These results taken together with some other recent observations (see Comments) suggest a possible role of this dilator mechanism in the control of the microcirculation and the transcapillary exchange

Methods Observations were obtained from 7 cats anesthetized with chloralose (50 mg/kg b w) and urethane (100 mg/kg b w) The experiments were performed on the vascular bed of the lower leg muscles The preparation was placed in a water filled plethysmograph (38 C) to permit volumetric determination of the capillary filtration coefficient Venous outflow of blood arterial inflow pressure (AP) venous outflow pressure (VP) small artery pressure (SAP) and small vein pressure (SVP) were continuously monitored (for technical details see Lundvall 1972) The severed ipsilateral lumbar sympathetic chain (*L4*—*L5*) was activated at 1—8 Hz via a bipolar electrode Supramaximal stimulation was used which was found to be evoked with stimulus characteristics of 5 V and 5 ms duration Dibozane (*1 4 bis* (1 4 benzodioxan— 2 yl methyl) piperazine McNeil Laboratories Inc) dissolved in *phosphoric* or lactic acid (0 15—0 30 %) was slowly infused intra arterially to the muscle region to block selectively the α adrenoceptors (Viveros *et al* 1968) The venous *outflow of blood* during the infusion was discarded to avoid systemic effects and compensated for by transfusion from a donor animal

Results Control stimulation of the sympathetic nerves after cholinergic blockade (0 5—1 mg/kg b w atropine i v) evoked a well maintained constriction of the resistance vessels graded in relation to the excitation rate (~ 600 % resistance increase at 8 Hz) The constrictor response to nerve stimulation was usually abolished after Dibozane administration in doses > 75 μg/kg muscle and stimulation then evoked a clearcut dilatation at rates exceeding 1 Hz at 2—4 Hz the

dilatation was usually maintained during the 5 min excitation period whereas at 8 Hz the initial dilator response was often followed by a slight constriction The dilator effect was related to the stimulation frequency and vascular resistance decreased below the prestimulatory level by about 15 25 and 35 % at 2 4 and 8 Hz respectively These responses were not mediated via the sympathetic cholinergic dilator fibres since atropine in adequate dose was given initially in the experiments and since the dilatations were unaffected by additional atropine (0 5 mg/kg b w) The dilator effects were however completely eliminated by propranolol (100—400 μg/kg b w intravenously) showing that they were caused by β adrenoceptor activation Mention should be made that Dibozane as well as the solvent *per se* caused some decrease of vascular resistance The described dilator effects were however, obtained after the infusion of Dibozane when vascular tone had returned to the control level

To obtain more detailed information about the localization of the described neurogenic β adrenergic dilator response an analysis of resistance changes in delimited segments of the vascular tree (segmental resistances) was performed from recordings of blood flow and of blood pressure at various points from artery to vein Figures could then be derived for total vascular resistance (R_T) in the muscle region [(AP VP)/flow] for proximal arterial resistance [(AP SAP)/flow] small vessel resistance [(SAP SVP)/flow] and for venous resistance [(SVP VP)/flow] (*cf* Lundvall 1972) The results of this analysis were consistent and showed that the β adrenergic dilatation predominantly occurred in the small resistance vessels This may be illustrated by the following data from a representative experiment In the control period AP was 97 SAP 48, SVP 16 and VP 5 mmHg and blood flow was 9 1 ml/min × 100 g tissue R_T was thus 10 1 mmHg/(ml/min × 100 g) and the consecutive segmental resistances 5 4 3 5 and 1 2 mmHg/(ml/min × 100 g) The magnitude of the control SAP value indicates that it was monitored from a relatively distal point in the arterial vascular tree As a result of sympathetic stimulation at 4 Hz in the Dibozane blocked region R_T decreased to 7 1 mmHg/(ml/min × 100 g) *i e* by 30 % Almost 80 % of this resistance decline was due to a decrease of small vessel resistance which was reduced to 1 2 mm Hg/(ml/min × 100 g) or by 66 % Some resistance decline also occurred in the proximal arterial vessels (11 %) and in the venous section (9 %)

After α adrenergic blockade sympathetic stimulation evoked a relaxation of the precapillary sphincters evidenced as an increase of the capillary filtration coefficient by 15 to 45 % This effect was markedly reduced or abolished by propranolol at all stimulation rates indicating that it was mainly caused by β receptor activation

Comments The present study shows that a β adrenergic dilator response can be evoked in the cat skeletal muscle during sympathetic stimulation and that it predominantly is confined to resistance vessels of small diameter and to precapillary sphincters Such a micro-vascular dilatation with its moderate influence on total blood flow might primarily if evoked during sympathetic activation in the i

organism serve to improve capillary (nutritional) exchange and could be of special importance if *there is a simultaneous α adrenergic constriction reducing regional* blood supply. Apparently this dilator response is quite different from that evoked by the sympathetic cholinergic dilator system which mainly seems to control the somewhat larger resistance vessels (*e g* Djojosugito *et al* 1968 Folkow Sonnenschein and Wright 1971) and thereby total blood flow. Direct support for the suggested neurogenic β-effect on capillary exchange has been obtained in preliminary experiments which show that the transcapillary fluid absorption occurring in *response to sympathetic stimulation is reduced by some 50 % after β adrenoceptor* blockade. This finding could mainly be related to abolition of a β adrenergic relaxation of precapillary sphincters and hence to a reduction of the capillary surface area available for fluid exchange.

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References

DJOJOSUGITO A. M B FOLKOW B LISANDER and H. SPARKS Mechanism of escape of skeletal *muscle resistance vessels from the influence of sympathetic* cholinergic vasodilator fibre activity *Acta physiol scand* 1968 *72* 148—156

FOLKOW B R R. SONNENSCHEIN and D L WRIGHT Loci of neurogenic and metabolic effects on precapillary vessels of skeletal muscle *Acta physiol scand* 1971 *81* 459—471

LUNDVALL J Tissue hyperosmolality as a mediator of vasodilatation and transcapillary fluid flux in exercising skeletal muscle *Acta physiol scand* 1972 Suppl 379

TUTTLE R S and G K MOE Reflex beta adrenergic vasodilatation in the cat. *Amer J Physiol* 1973 *225* 402—407

VIVERO O H D G GARLICK and E M. RENKIN Sympathetic beta adrenergic vasodilatation in skeletal muscle of the dog *Amer J Physiol* 1968 *215* 1218—1225

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From the Department of Pharmacology University of Lund Sweden

Sarcomere Lengths at the Peak of the Length-Tension Curve in Living and Fixed Rat Papillary Muscle

by

ARTHUR F GRIMM[1] AND BJORN WOHLFART

Striated muscle force production depends upon muscle length The pioneering work of Blix (1892) clearly demonstrated this for skeletal muscle, while the later work of Frank (1895) and Starling (1918) extended this relationship to the whole heart The work of Lundin (1944) showed that the isolated papillary muscle can be used for mechanical studies of myocardium The demonstration by Gordon Huxley and Julian (1966) and Edman (1966) related the length dependence of striated muscle to ultrastructural events within the sarcomere of amphibian skeletal muscle Similar studies have extended the length tension relationship to the myocardium (Grimm and Whitehorn 1968 Grimm *et al* 1970 Sonnenblick *et al* 1963) Unfortunately these myocardial sarcomere studies have relied upon histologic material It is well recognized that fixation dehydration and embedding introduce length distortions The recent demonstration by Pollack (1973) showed the feasibility of using water immersion light microscopy on the isolated living papillary muscle to directly measure myocardial sarcomere lengths The present study reports on sarcomere lengths in the unstimulated living and in the fixed rat right ventricular papillary muscle at optimum length for force production

Male albino rats of the Sprague Dawley strain weighing between 150 and 200 g were used Both female and heavier rats were also used Neither sex nor body weight seem to influence the sarcomere length at optimum muscle length. A right ventricular papillary muscle was dissected free without touching the muscle with the dissecting instruments The muscle was tied by Ethicon Mersilene braided 6/0 suture material to platinum mounting loops both at the tendon attachments and at a block of septal myocardium removed with the muscle The muscle was mounted in a horizontal position in a specially constructed muscle chamber as previously described (K E Andersson *et al* 1974) The compliance of the transducer was 1.3 µm/mN The muscle was maintained at a temperature of 36.5 C in a continuously flowing solution oxygenated with 95 % O_2 and 5 % CO_2 of the following composition NaCl 100 mM KCl 4 mM $NaHCO_3$ 0 mM $MgSO_4$ 1.5 mM NaH_2PO_4 1.5 mM $CaCl_2$ 4 mM Na acetate 20 mM Glucose 10 mM insulin 4 IE/l During the equilibration period of 20 min the muscle contracted isometrically with a preload of 3 mN Using platinum plate electrodes the stimulation frequency was 0.5 Hz with a pulse duration of 2 ms and an intensity of 50 % above threshold The muscle length was adjusted in steps of 0.05 mm to get maximum force production (6—10 mN) The average length and greatest diameters of these tapering muscles were 1.[illegible]—2.5 mm and 0.5—1.0 mm, respectively as directly measured optically The muscle thickness though not directly measured was estimated to

[1] Address Departments of Histology and of Physiology Colleges of Dentistry and of [illegible] University of Illinois at the Medical Center Chicago Illinois 60612 U.S.A.

TABLE I Sarcomere lengths (mean ± SD) in μm

Exp Code	Living muscle			Fixed muscle	
	n		Whole muscle n = 12	n	Homogenized
83	20	2 38 ± 0 04			
94	30	2 37 ± 0 06			
95	30	2 38 ± 0 06		30	2 25 ± 0 080
96	30	2 39 ± 0 05		30	2.28 ± 0 096
99	30	2 41 ± 0 05			
91	10	2 35 ± 0 05		30	2 32 ± 0 099
		2 384 ± 0 004*	2 283 ± 0 106		2 284 ± 0 010

n = number of measurements
* Standard error of mean

be 1/4—1/5 of the muscle width The muscles were photographed on Kodak Plus-X pan film with an Asahi Pentax camera. The camera was mounted via a Zeiss adapter on a microscope with a water immersion lens (40/0 NA. 0 75 160/ Zeiss) The illuminating light was passed through a green filter During the photography the muscle was left unstimulated but was allowed to reestablish stability in force production before the next stimulation break The films were analyzed with a Nikon model 6C comparator at 20 X magnification. The length of a row of 10 sarcomeres was measured Total magnification was about 900 X. Values are reported as single sarcomere lengths To insure sampling each exposure was taken at either a different focal plane or in a different area of the muscle A maximum of 3 measurements were taken from a single photographic image Measurements were made independently by 3 persons with no real differences between the results Between 10 and 30 measurements were made on each of 6 right ventricular papillary muscles 4 of the 6 muscles were fixed at the end of the experiment These muscles were arrested with an isotonic high potassium solution (150 mM) for 20 min photographed and then exposed to continuously flowing 10 % formalin and again photographed after 30 min of fixation

K arrest produced an initial rise and subsequent fall in tension as described by Morad (1969) Microscopic examination revealed cellular activity (shivering) even at a time when tension production was 0 At the end of the arresting period no activity was observed Sarcomeres measured at this time were identical in length to those measured between mechanical contraction.

Fixation with formalin as directly observed was a rapid process the muscle lost its transparency within a minute and sarcomeres were far more difficult to observe During fixation tension initially increased 1—2 mN By the end of the fixation period of 30 min tension had returned very nearly to initial values Photography was greatly limited in its choice of areas where sarcomeres were visible

The fixed muscle was gently and coarsely hand homogenized in a ground glass homogenizing tube The homogenate which consists of cells or large fragments of cells was mixed with glycerol jell and placed on a microscope slide and covered with a coverslip Under these conditions the rows of sarcomere lie essentially within the focal plane of the microscope The measurement of the length of 10 sarcomeres was made with the use of an ocular micrometer at 1 000 X magnification The sarcomeres were thus directly measured without the usual dehydration and embedding steps of histologic methodology The latter procedures were carried out by S H at the University of Illinois at the Medical Center

The uncertainty in the muscle length determination did not exceed 0 05/20 0 mm = 2.5 % The uncertainty due to the optical limitations of the equipment (the resolution power of this system about 0 4 μm is about 2 of the length of 10 sarcomeres (23 8 μm)

The average sarcomere length of the right ventricular papillary muscle was 2 384 μm (Table I) Since there were no real differences between muscles the muscle measurements were pooled and the S E determined (0 004 μm) The direct measurements of the formalin fixed sarcomeres revealed a length of 2 283 ± 0 106

μm A criticism of our present values might be that our sampling technique favoured sarcomeres located at the periphery of the muscles Since an examination of the homogenized muscles reveals an almost identical sarcomere length with a small standard deviation (0 010 μm) for the fixed material and since the latter technique sampled from the entire muscle we conclude that sarcomere lengths are homogeneous and uniform throughout the muscle at optimum length From our results we can also estimate the shrinkage induced by 10 % formalin to be (2 384—2 284)/2 384 = 4 2 % The sarcomere lengths at optimum length do exceed the estimate of Grimm *et al* (1970) However they used indirect methods to estimate the shrinkage induced by formalin Applying their method of readjusting muscle length to maintain the prefixation tension we too find minimal shrinkage with formalin It should be noted that the variability in measurements as reflected by the standard deviations increased two fold from the living to the fixed material The results do suggest that the fixing+homogenizing procedure is reproducible and of experimental value

In conclusion we find that sarcomere lengths of the unstimulated right ventricular papillary muscle of the rat at optimum muscle length compose a very homogenous population with a mean sarcomere length of 2 384±0 004 μm In addition formalin fixation produces sarcomere shrinkage of about 4 2 %

Since the submission of the present manuscript the paper by Pollack and Huntsman Amer J Physiol 1974 227 383—389 has appeared These authors also studied sarcomere lengths in living mammalian cardiac muscle Their conclusion that the physiological range of sarcomere length is variable from muscle to muscle is certainly at variance with our conclusions

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References

Andersson K E and K A P Edman Effects of lanthanum on potassium contractures of isolated twitch muscle fibres of the frog *Acta physiol scand* 1974 *90* 124—131
Blix M Die Lange und die Spannung des Muskels *Skand Arch Physiol* 1892 *3* 295—318
Edman K A P The relation between sarcomere length and active tension in isolated semitendinosus fibres of the frog *J Physiol (Lond)* 1966 *183* 407—417
Frank O Zur Dynamik des Herzmuskels *Z Biol* 1895 *32* 370
Gordon A M A F Huxley and F J Julian The variation in isometric tension with sarcomere length in vertebrate muscle fibers *J Physiol (Lond)* 1966 *184* 170—192
Grimm A F K V Katele R Kubota and W V Whitehorn Relation of sarcomere length and muscle length in resting myocardium *Amer J Physiol* 1970 *218* 1412—1416
Grimm A F and W V Whitehorn Myocardial length tension sarcomere relationship *Amer J Physiol* 1968 *214* 1378—1387
Leupin G Mechanical Properties of Cardiac Muscle *Acta physiol scand* 1944 *7 Suppl* 20 7—86
Morad M Contracture and Catecholamines in Mammalian Myocardium *Science* 1 166 505—50
Pollack F H and W G Guntheroth Active force sarcomere length relations in ... cardiac muscle *Fed Proc* 1973 *32* 1189
Sonnenblick E H D Spiro and T S Cottrell Fine structural changes in heart muscle in relation to the length-tension curve *Proc Nat Acad Sci (Wash)* 1963 *49* 193—200
Starling E H *The Linacre Lecture on the Law of the Heart* (Cambridge 1915) York Longman Green 1918

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From the Research and Development Laboratories Astra Lakemedel AB Sodertalje Sweden

On the Fate of Dopamine-ß-Hydroxylase after Release from the Pheripheral Sympathetic Nerves in the Cat

By

SVANTE B ROSS, HANS E ERIKSSON and WERA HELLSTROM

The enzyme dopamine β hydroxylase (DBH) which catalyzes the hydroxylation of dopamine to noradrenaline is located in the storage vesicles of the sympathetic nerves and the chromaffin cell in the adrenal medulla and is released from these sites together with noradrenaline (Geffen *et al* 1969, de Potter *et al* 1969 Viveros *et al* 1969) Since blood serum contains appreciable amounts of DBH activity it has been suggested that the enzyme relased from the sympathetic nerves and the chromaffin cells reaches the blood circulation (Weinshilboum and Axelrod 1971 a b) DBH is a large molecule with a molecular weight of about 290 000 (Friedman and Kaufmann 1965) and may therefore only slowly pass from the interstitial fluid in which it is released directly into the blood circulation The lymphatic system appears to be an alternative route for DBH to the blood Recently the occurrence of DBH in human lymph was reported (Åberg *et al* 1974) This observation does not by itself prove that the DBH in the lymph emanates from the sympathetic nerves since plasma proteins are normal constituents of the lymph In order to examine whether the DBH in lymph is derived from the sympathetic nerves we have now determined the effect of stimulation of the sympathetic nervous system in anesthetized cats on the DBH activity in lymph and plasma The ganglionic stimulatory agent dimethylphenylpiperazinium iodide (DMPP) was used for a massive stimulation of the sympathetic nervous system

6 cats weighing 2 5—4 0 kg anesthetized with sodium pentobarbitone were used in the experiments The thoracic duct was cannulated just below the diaphragm Lymph was collected in ice chilled tubes for periods of 30 min The lymph flow was determined by measuring the volume of the lymph 3 consecutive doses of DMPP 0 1 mg/kg were injected into the femoral vein at intervals of 10 min The first injection was given 1 h after the start of the lymph collection Blood samples were taken from the femoral vein every 30 min The blood pressure was measured in the femoral artery The DBH activity in plasma and lymph was measured according to the method of Molinoff *et al* (1971) using tyramine as the substrate with the modification that the DBH assay was performed at pH 5 2 (Weinshilboum *et al* 1973) The plasma and lymph were diluted with 8 volumes of distilled water before the assay The optimal copper sulphate concentration for the DBH assay of both plasma and lymph was 7×10^{-6} M The DBH activity was expressed in normal octopamine formed per ml lymph per 20 min incubation The amount of the DBH in the lymph collected for periods of 30 min was also estimated

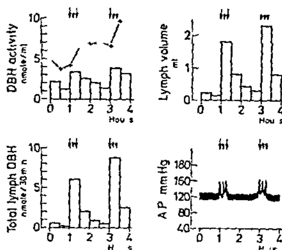


Fig 1 Effects of stimulation of the sympathetic nervous system by dimethylphenylpiperazinium iodide (DMPP) on the levels of DBH in plasma (x) and lymph (staples) total output of DBH in lymph lymph flow and arterial blood pressure (A P) in a cat (4 0 kg) anesthetized with sodium pentobarbitone Blood samples were taken every 30 min and the lymph was collected from the thoracic tract just below the diaphragm during periods of 30 min The arrows indicate injections of 0 1 mg/kg i v of DMPP DBH was analyzed according to the method of Molinoff *et al* (1971) The DBH activity is expressed in nmole octopamide formed per ml per 20 min incubation

Cat plasma and lymph contained detectable amounts of DBH activity (Table I) The plasma DBH level varied considerably more than that observed in the albino (Sprague Dawley) rat (unpublished observation) and was hundred folds lower than the mean DBH level in man (Weinshilboum and Axelrod 1971 a Åberg *et al* 1974) In 4 of the 6 cats examined the DBH activity in lymph was lower than that in plasma Stimulation of the peripheral sympathetic nervous system with DMPP caused a marked increase in the DBH activity in lymph and plasma in all cats (Table I) The lymphatic flow was also increased by DMPP and the total amount of DBH collected after DMPP was therefore largely increased However the lymph DBH activity returned rather rapidly to normal values after the stimulation whereas the plasma DBH remained elevated during a considerably longer period (Fig 1)

TABLE I Effects of dimethylphenylpiperazinium iodide (DMPP) on dopamine β-hydroxylase in lymph and plasma and on the lymph flow in 6 anesthetized cats The lymph was collected from the thoracic duct immediately below the diaphragm The values are means ± S E with the range in brackets 1 h before and 1 h during and after DMPP (3 × 0 1 mg/kg i v with intervals of 10 min)

	Lymph DBH nmol/ml	Lymph Total DBH nmol/h	Lymph Volume ml/h	Plasma DBH nmol/ml
Before DMPP	3 6 ± 0 6 (1 7 – 6 0)	9 6 ± 3 8 (0 6 – [illegible]7 6)	2 [illegible] ± 0 6 (0 4 – 4 6)	5 8 ± 1 3 (1 2 – 10 3)
During and after DMPP	5 1 ± 0 6 (3 0 – 6 7)	24 0 ± 8 5 (7 8 – 61 6)	4 3 ± 1 1 ([illegible] 0 – 9 [illegible])	9 ± [illegible] 0 (2 2 – 16 1)
Increase	1 5 ± 0 3 (0 7 – [illegible] 6)	13 7 ± 5 3 (4 4 – 34 0)	2 2 ± 0 6 (0 4 – 4 6)	2 1 ± 0 8 (0 9 – 5 8)

* nmol octopamine formed per ml per [illegible]0 min incubation

The influence of the elevation of the arterial blood pressure on the DBH activity and the lymph flow was examined by injection of 1 noradrenaline in a dose (3 × µg/kg i.v.) which produced the same elevation of the blood pressure as that caused by DMPP No effects on the lymph volume and the lymph DBH were observed

The results of the experiments demonstrate a direct relationship between the stimulation of the sympathetic nervous system and the level of DBH activity in lymph and plasma The observation that the lymph DBH returned to the normal level after the stimulation much more rapidly than the plasma DBH indicates that lymph DBH does not simply reflect the plasma level of the enzyme On the contrary the difference of the time courses supports the hypothesis that the lymph DBH is elevated before the plasma DBH The large increase of the total output of lymph DBH during the sympathetic nerve stimulation probably explains the elevation of the plasma DBH although it cannot be excluded that a part of the latter may be derived directly to the blood circulation The lymph collected from the thoracic duct was only a part of the total amount of lymph delivered to the blood mainly that from the hind legs and the visceral part of the body While this work was in progress Ngai *et al* (1974) reported that the DBH activity in dog lymph was elevated after sympathetic stimulation The present work confirms this observation

References

ABERG H E H E HANSSON L WETERBERG S B ROSS and Ö FRODEN Dopamin β hydroxylase in human lymph *Life Sci* 1974 *14* 65—71

GEFFEN L P B G LIVETT and R A RUSH Immunological localization of chromogranins in sheep sympathetic neurones and their release by nerve impulses *J Physiol* (Lond) 1969 *204* 58P—59P

FRIEDMAN S and S KAUFMAN 3 4 Dihydroxyphenylalkylamine β hydroxylase a copper protein *J biol Chem* 1965 *240* PC552—PC554

MOLINOFF P B R WEINSHILBOUM and J AXELROD A sensitive enzymatic assay for dopamine β hydroxylase *J Pharmacol exp Therp* 1971 *178* 425—431

NGAI S H W DAIRMAN M MARCHELLE and S SPECTOR, Dopamine β hydroxylase in dog lymph —effect of sympathetic activation. *Life Sci* 1974 *14* 2431—2439

DE POTTER W P A F DE SCHAEPDRYVER A F MOERMAN and A D SMITH Evidence for the release of vesicle proteins together with noradrenaline upon stimulation of the splenic nerve *J Physiol* (Lond 1969 *204* 102P—104P

VIVEROS O H L ARQUERO and N KIRSCHNER Release of catecholamines and dopamine β-oxidase from the adrenal medulla. *Life Sci* 1969 *7* 609—618

WEINSHILBOUM R and J AXELROD Serum dopamine β hydroxylase activity *Circulat Res* 1971 a *28* 307—315

WEINSHILBOUM R and J AXELROD Serum dopamine β hydroxylase *Decrease after chemical sympathectomy* *Science* 1971 b *173* 931—934

WEINSHILBOUM R M F A RAYMOND L R ELVEBACK and W H WEIDMAN Serum dopamine β hydroxylase activity Sibling-sibling correlation *Science* 1973 *181* 943—945